SYNTHETIC GENOMICS®

Figures and Tables for:

TSCA Experimental Release Application of Synthetic Genomics Inc. – Open Pond Research and Development

The green microalgal strain Parachlorella STR26155 engineered with green fluorescent protein (GFP) for environmental tracking

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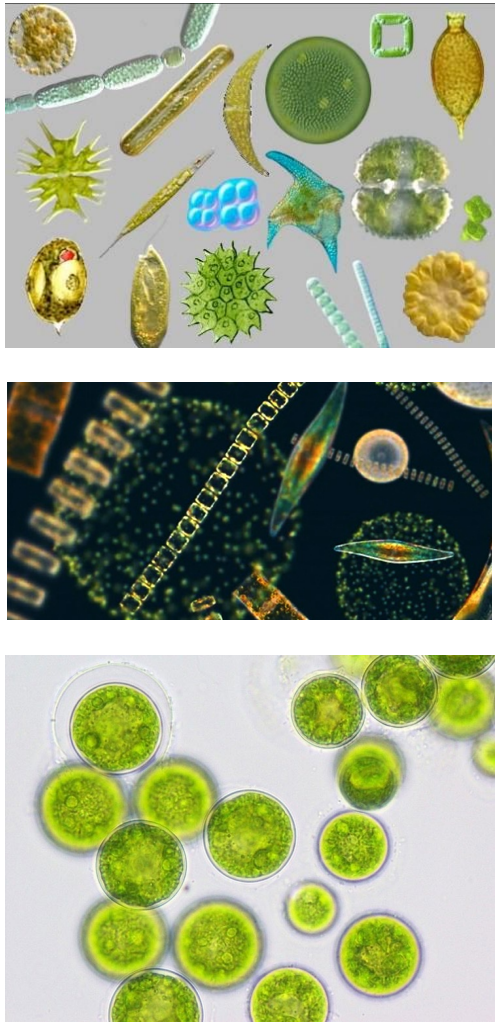
Figure INT 1: Images showing the incredible diversity of algal species found in nature (left). Pictograph of an algae chloroplast, exhibiting how algae can use carbon dioxide and sunlight to grow, reproduce, make oxygen, and store energy as fatty-acid oils and starches (right). Algae oils can be isolated and then converted into biodiesel or renewable diesel fuels in a refinery.



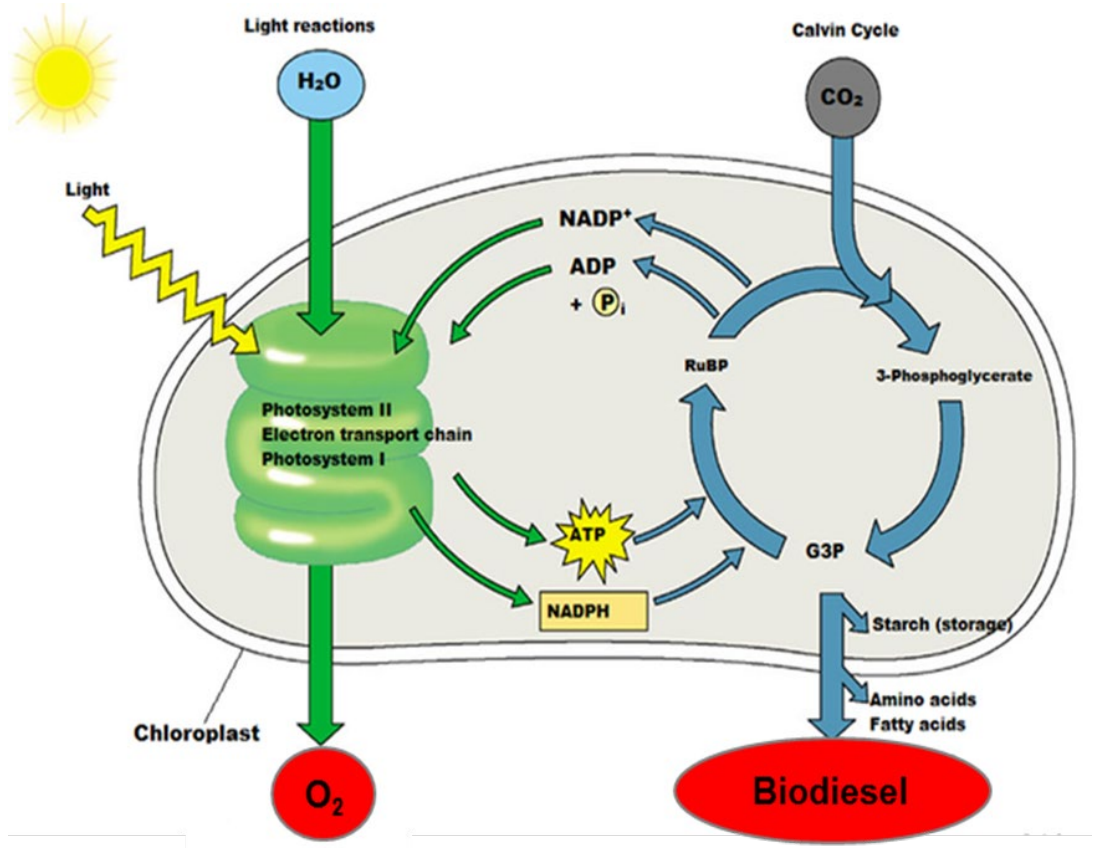
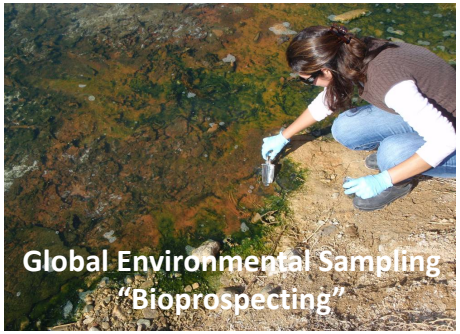
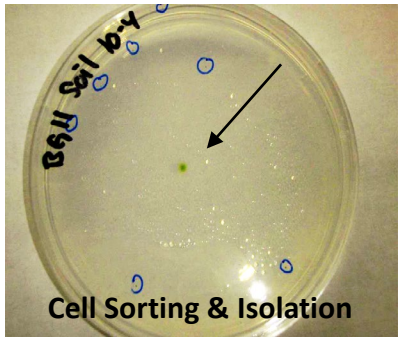


Figure INT 2: SGI's disciplined R&D methodology from discovery to scale-up.





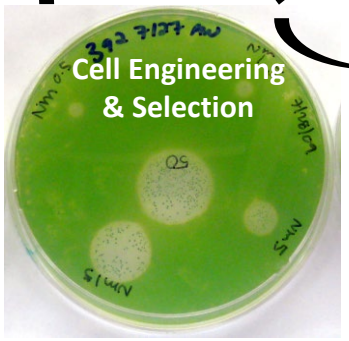
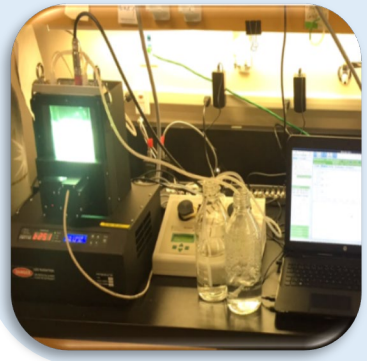


Figure INT 3: SGI's algae capabilities: Discovery and cell engineering through agronomics and scale-up.

Laboratory - La Jolla

- World-class microorganism engineering
- DNA sequencing & analytical labs
- Extensive culture collection from global sampling

- Environmental metagenomics
- Scale-down pond simulation
- Programmable daylight and temperature



- Photo-bioreactors (PBRs)
- Adaptive evolution
- On-line monitoring

Greenhouse - La Jolla

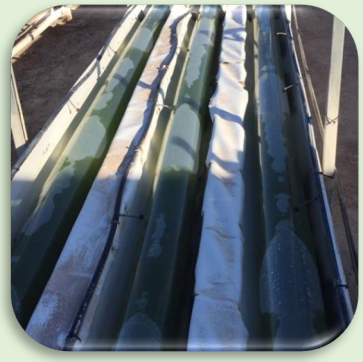
- BL1-LS Greenhouse w/ 2° containment
- Analytical and processing labs
- Chlorination system

- Mini-ponds 1.9 m^2 / 400 L
- Specialized PBRs
- On-line monitoring

- Reconfigurable ponds
  25 m^2 / 5,200 L
  75 m^2 / 16,000 L

Greenhouse & Outdoor - Calipatria

- BL1-LS green-house w/ 2° containment
- Indoor mini-ponds 3.4 m^2/ 700 L
- On-line monitoring



- Multiscale horizonal-tube PBRs, six BL1-LS w/2° containment
- Outdoor mini-ponds 1.9 m^2 / 400 L

- 7 x 1-acre race-way ponds 4000 m^2 / 1 MM L
- 2 x 0.1-acre GLSP ponds w/ 2° containment
- Weather station
- Environmental monitoring

Figure INT 4: Satellite image identifying the locations of SGI's R&D Facilities in La Jolla and Calipatria, CA.

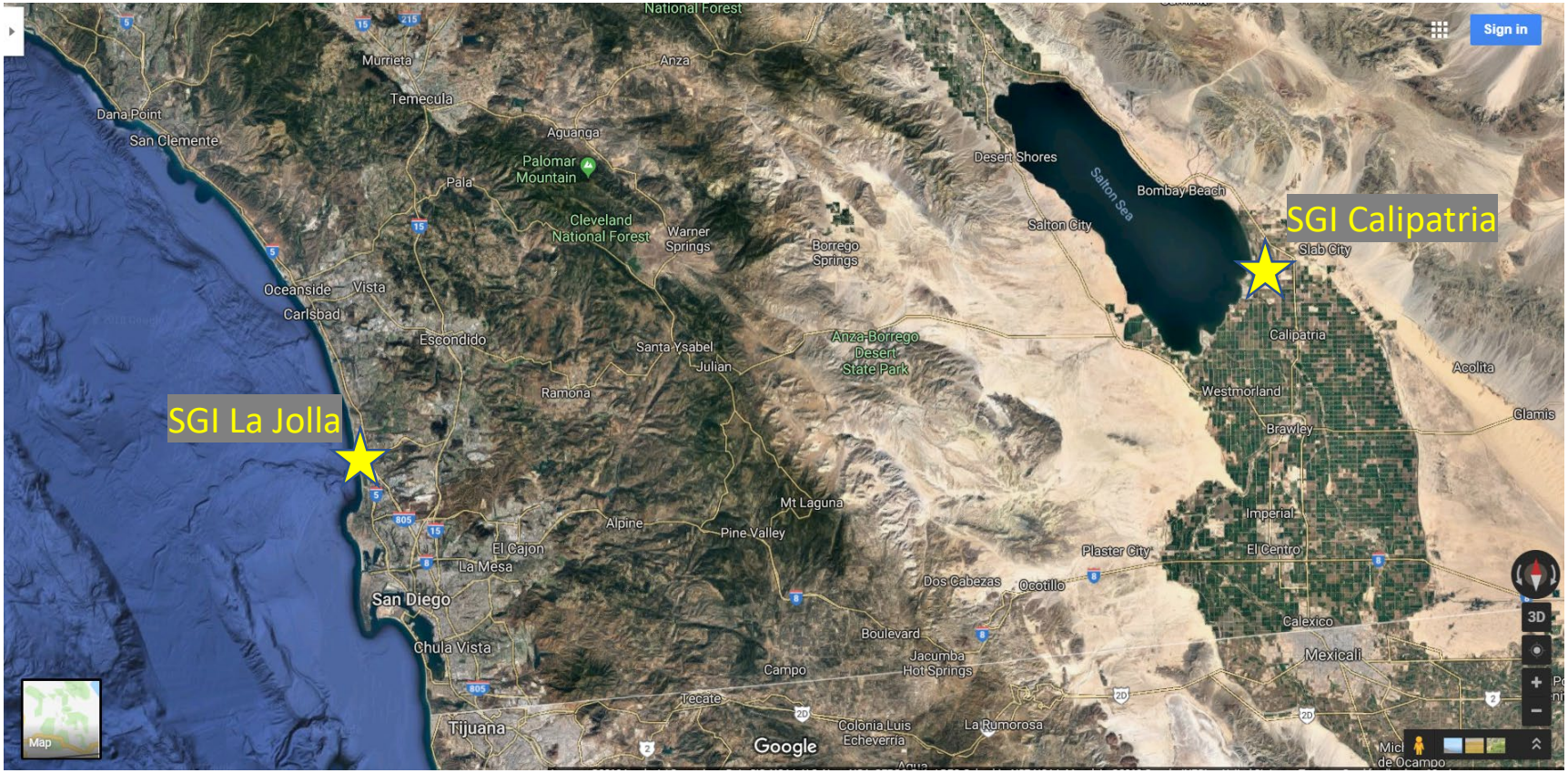


Figure INT 5: Aerial photo of the SGI California Advanced Algae Facility (CAAF) in Calipatria, California.

Figure A1: Neighbor-joining phylogenetic tree produced from an alignment of full-length 18S SSU rRNA sequences.

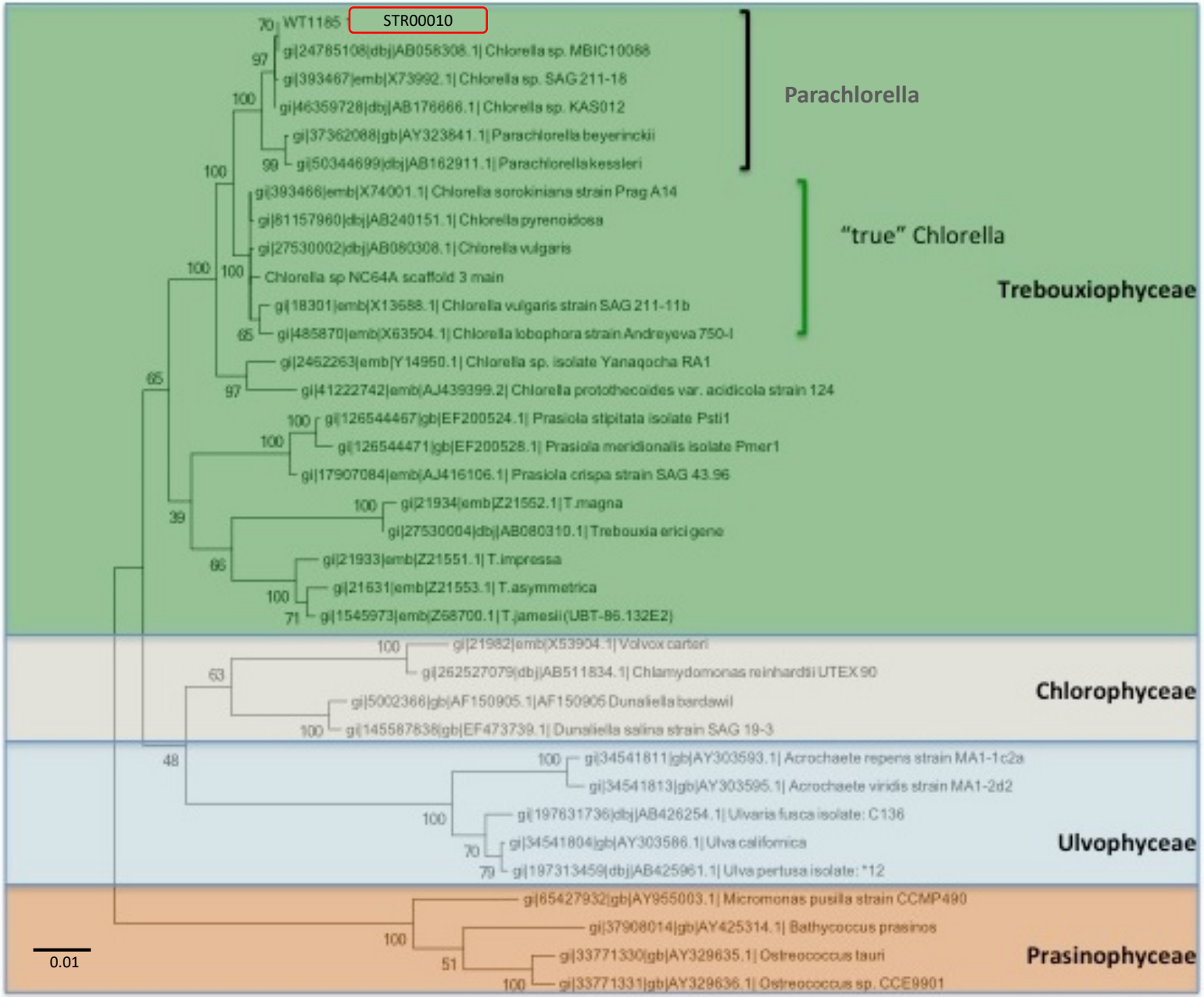
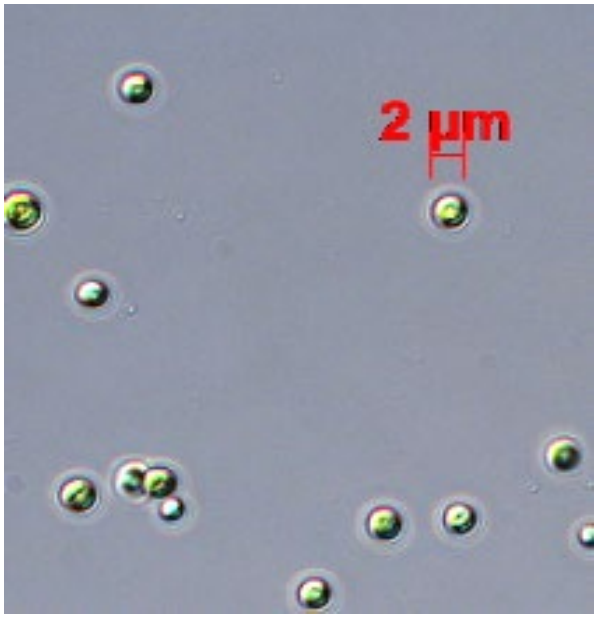
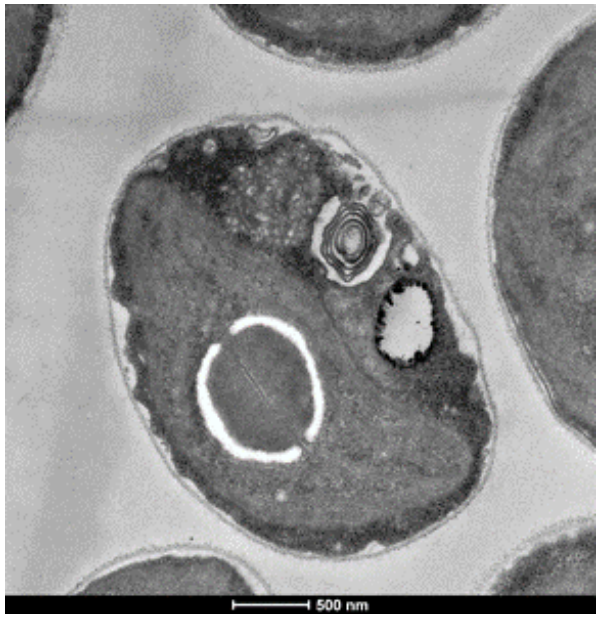


Figure A2: Light micrograph (A) and transmission electron micrographs (TEM) (B,C) of SGI *Parachlorella.*

(A) Light Micrograph



(B) Electron Micrograph Cross-Section



(C) Electron Micrograph Cross-Section

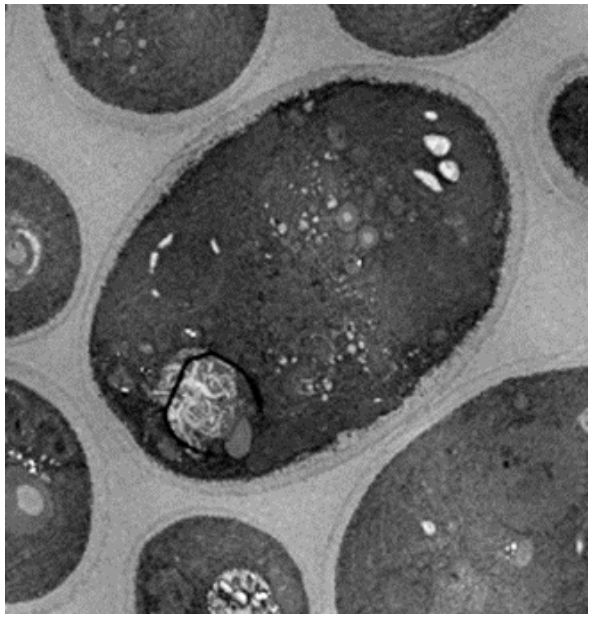


Figure A3: *Parachlorella* STR00012 measured growth rates under various salinity, temperature, pH and bicarbonate additions.

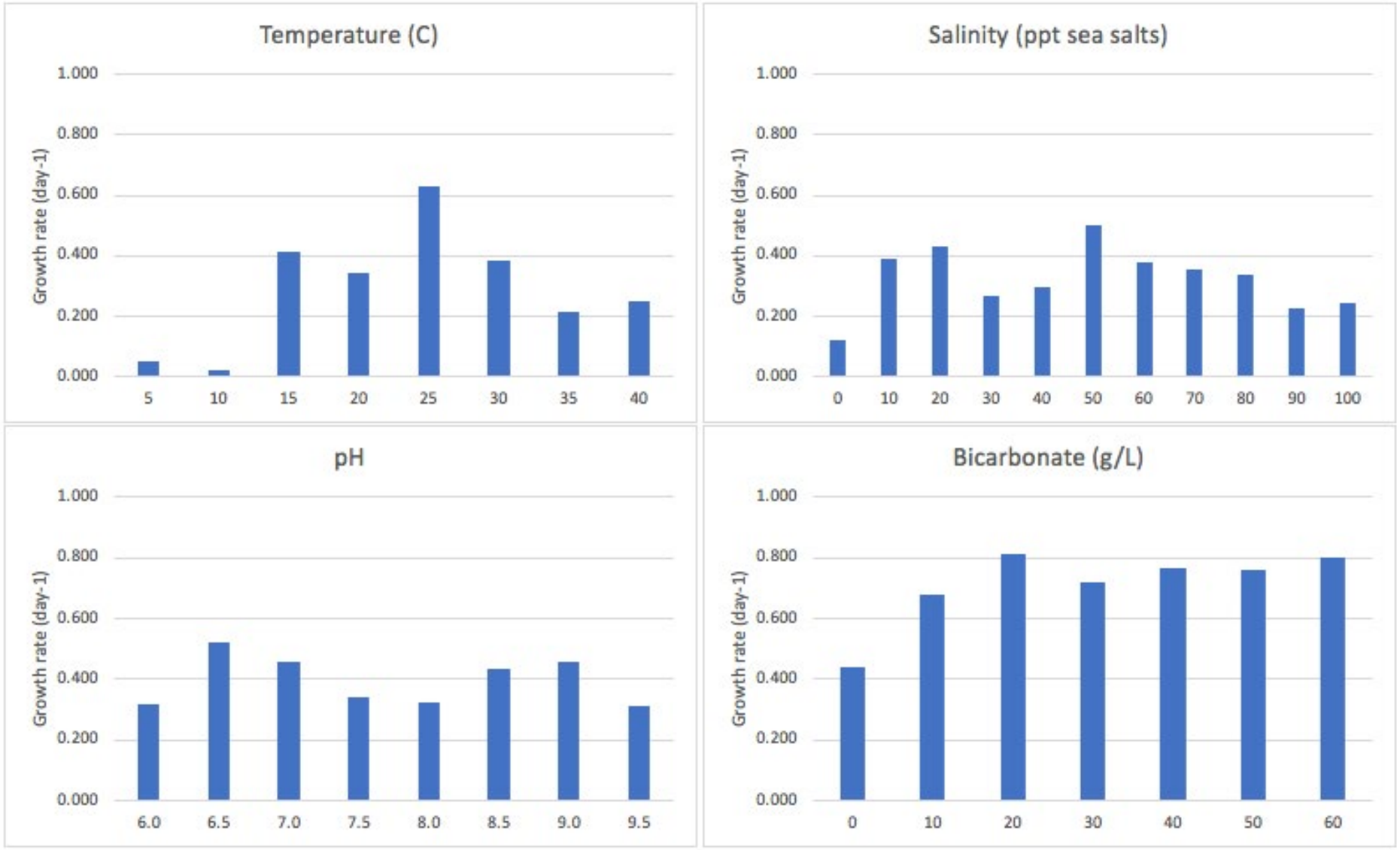


Figure A4: Approximate biomass composition (A) and lipid profile for *Parachlorella* STR00012.

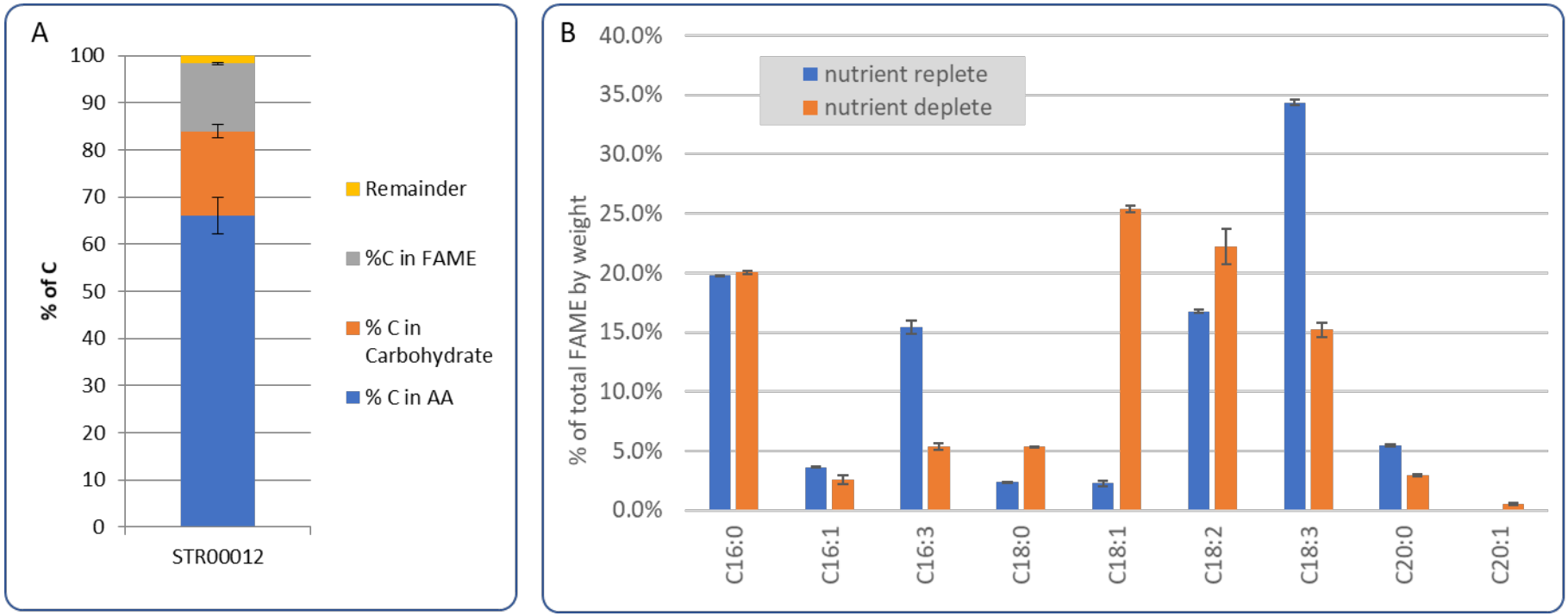


Figure C1: Flow diagram depicting major steps in strain construction

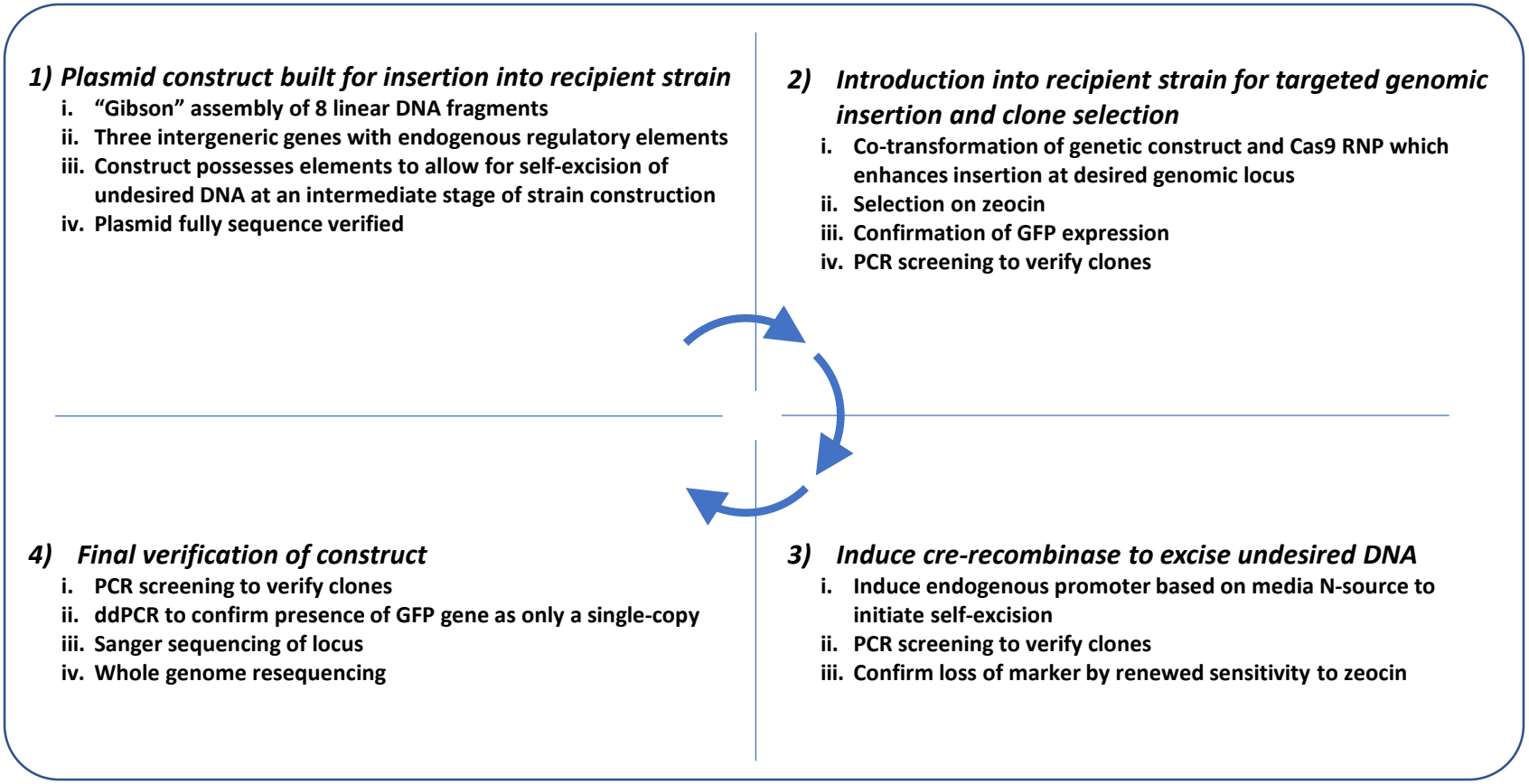


Figure C2: Plasmid map of NAS14335. Outer ring depicts genetic material present in strain during phases of construction. Cassette inserted into recipient strain genome depicted in blue. Following cre-recombinase mediated excision the portion remaining in the subject strain is denoted in green. Inner ring depicts the overlapping linear fragments detailed in the text, **Figure C3** and **Table C2**.

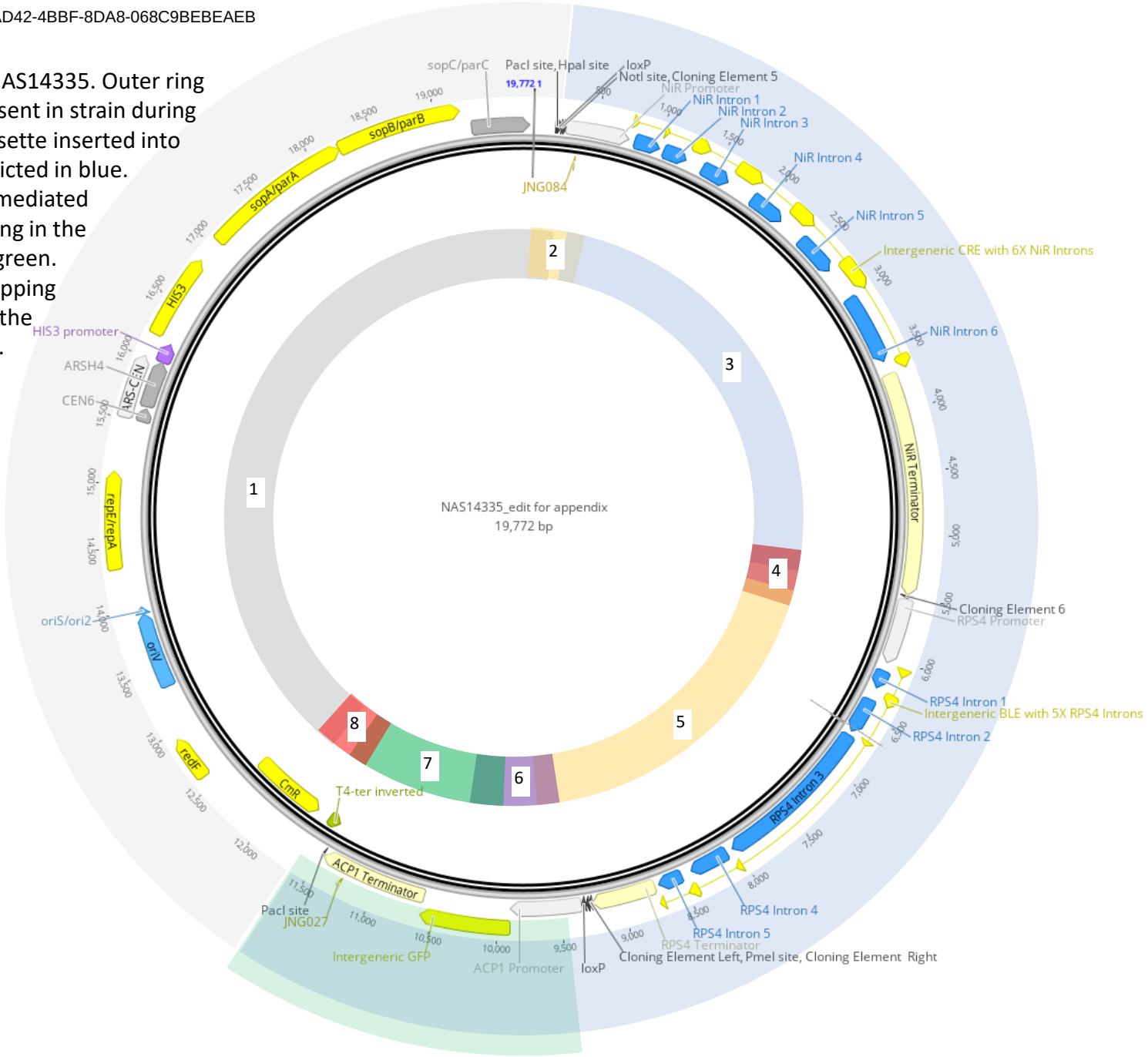


Figure C3: Detailed schematic of the eight linear DNA fragments used in the assembly of NAS14335.

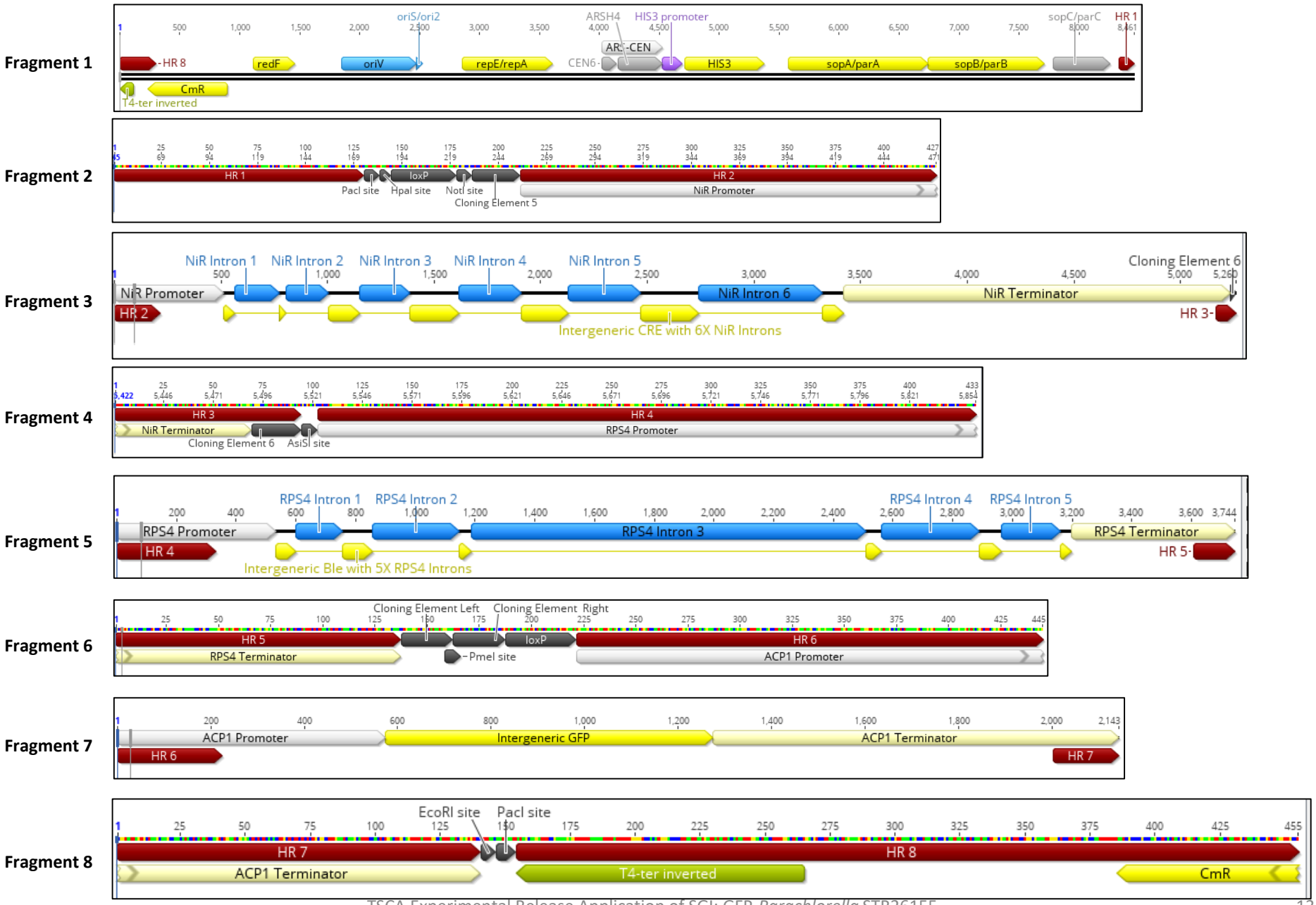


Figure C4: Graphical representation of RS1 locus in recipient strain STR0012 (top panel), intermediate locus prior to Cre-recombinase induction (middle panel), and final subject strain construction (bottom panel). Genomic regions with PCR priming locations depicted in orange to highlight the same region across panels. Green region indicates the inserted DNA which is retained in final subject strain.

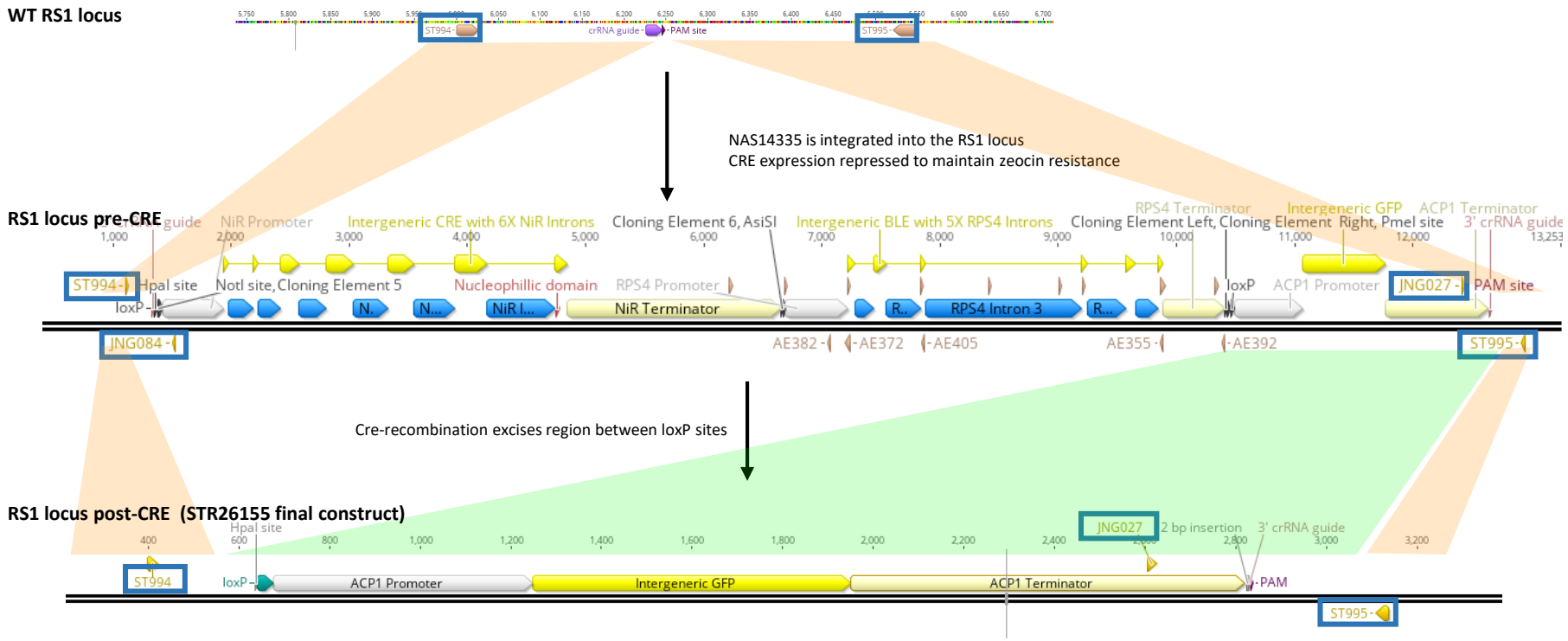
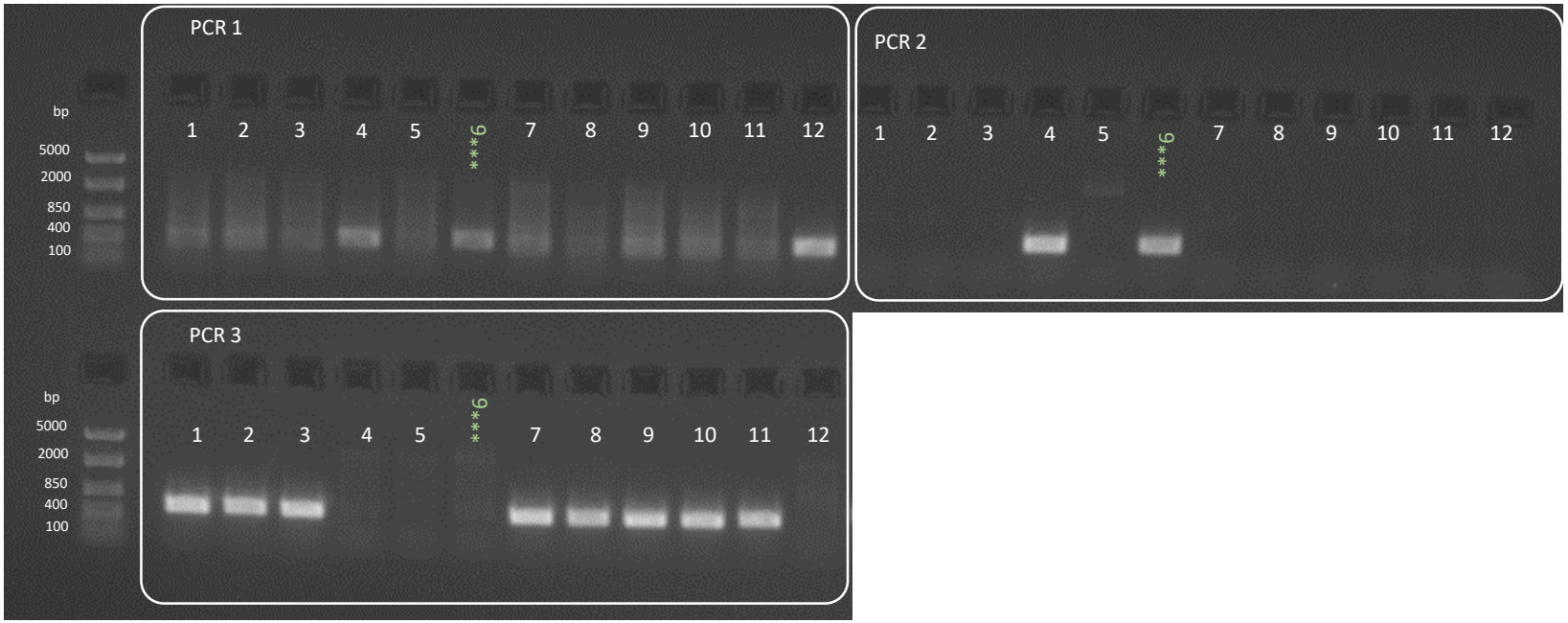


Figure C5: PCR screening results which verify the desired insertion cassette presence at genomic RS1 locus (top). Primer sequences and expected PCR product size provided (below). The same primer names are used in **Figure C4** to graphically show location. Clone 6 was selected for subsequent stages of strain construction.



PCR Primer Pair	Amplicon Description	Forward Primer	Forward Primer Sequence	Reverse Primer	Reverse Primer Sequence	Expected Amplicon for WT		
						WT	Pre-CRE Induction	Post-CRE induction
1	Genomic 5' of RS1 into NiR -promoter	ST994	CGTGGAGGGCTCA CCGGTTCTGGAG	JNG084	AACCTCAGCACTGCG CCTC	No band	416bp	No band
2	ACP1-terminator into genomic 3' of RS1	JNG027	GAATCCAGTTGAA ATTACGGCG	ST995	AGCTCCAGGCATCAC TTGCGGAGTCAGTG	No band	531bp	531bp
3	RS1 locus	ST994	CGTGGAGGGCTCA CCGGTTCTGGAG	ST995	AGCTCCAGGCATCAC TTGCGGAGTCAGTG	550bp	12kb+ (no band expected)	3kb

Figure C6: PCR screening results which verify loss of BLE and CRE at genomic RS1 locus insertion. Primer sequences and expected PCR product size provided in **Figure C5**. The same primer names are used in **Figure C4** to graphically show location. Isolate selected for final strain construct labeled as STR26155.

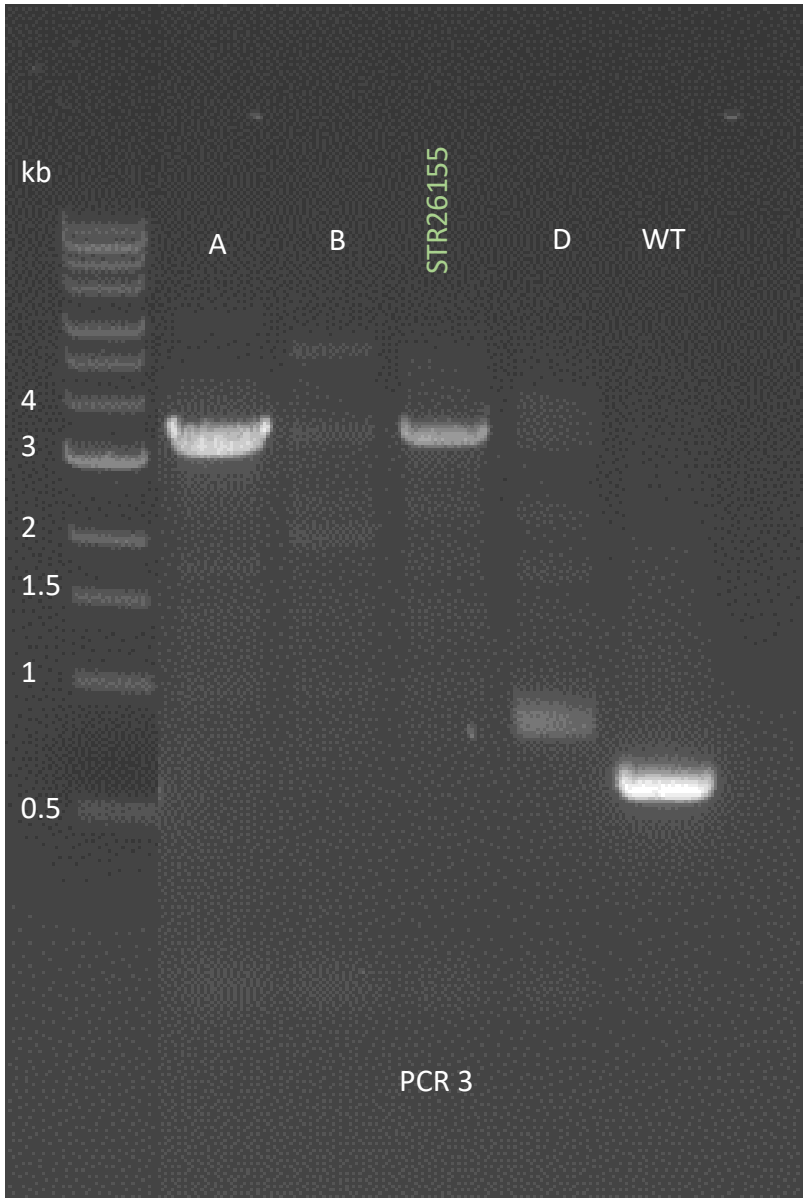


Figure C7: Confirmation of renewed sensitivity to zeocin following excision of CRE and BLE. Patch 15 represents STR26155.

Nitrate **Nitrate + Zeocin**

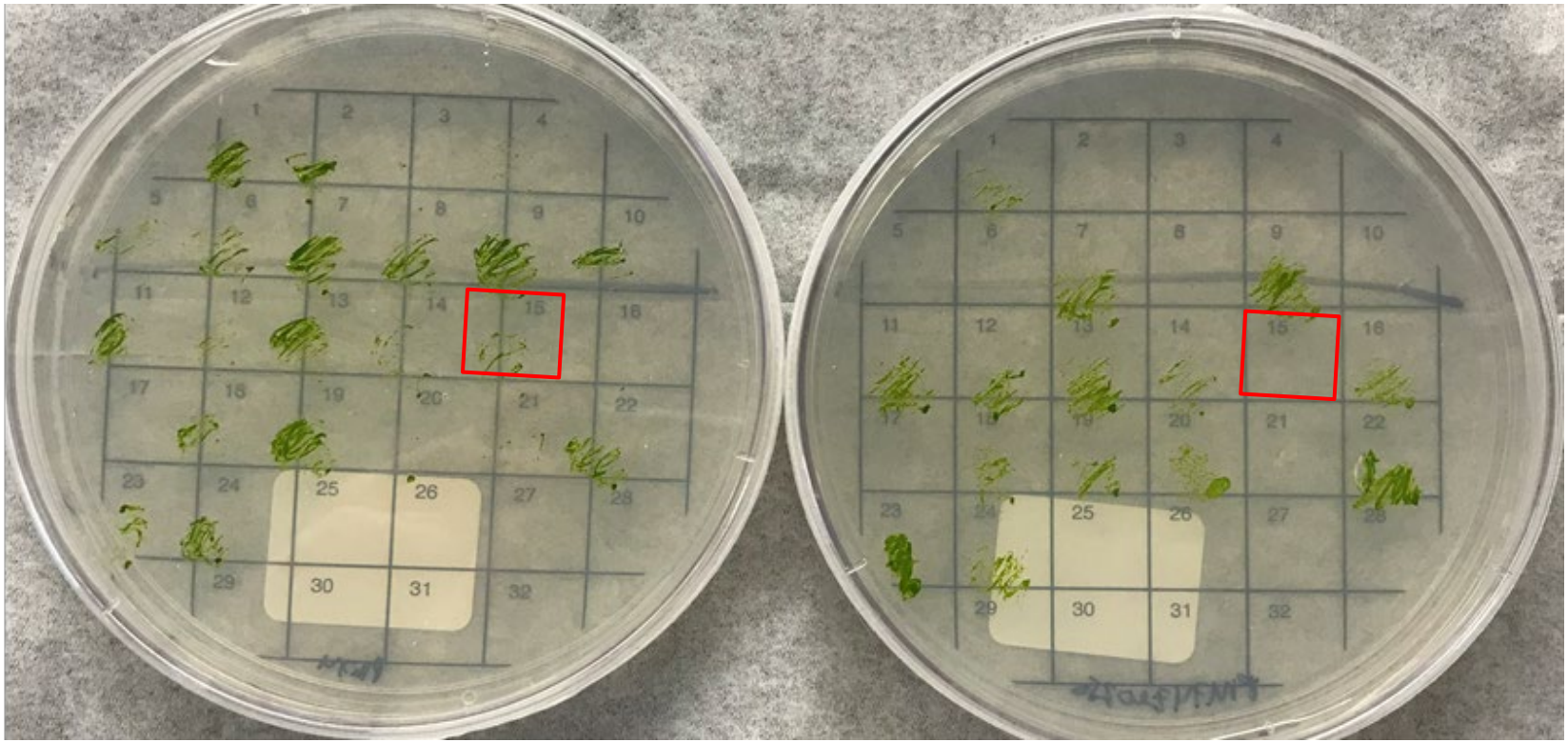


Figure C8: Schematic of final genetic construct and closeup to provide nucleotide level detail of 3’ end of insertion cassette.

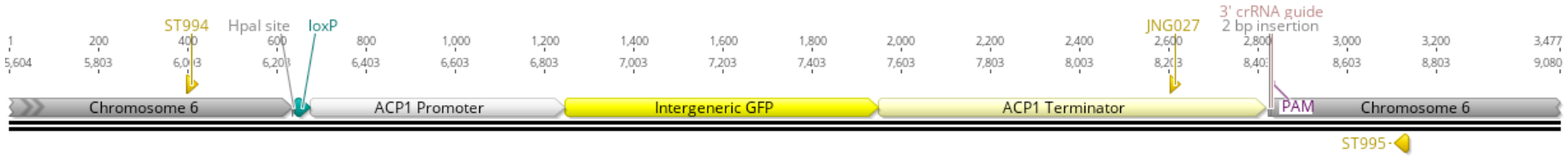


Figure C9: Droplet digital PCR for copy number verification of intergeneric genes used in subject strain construction.

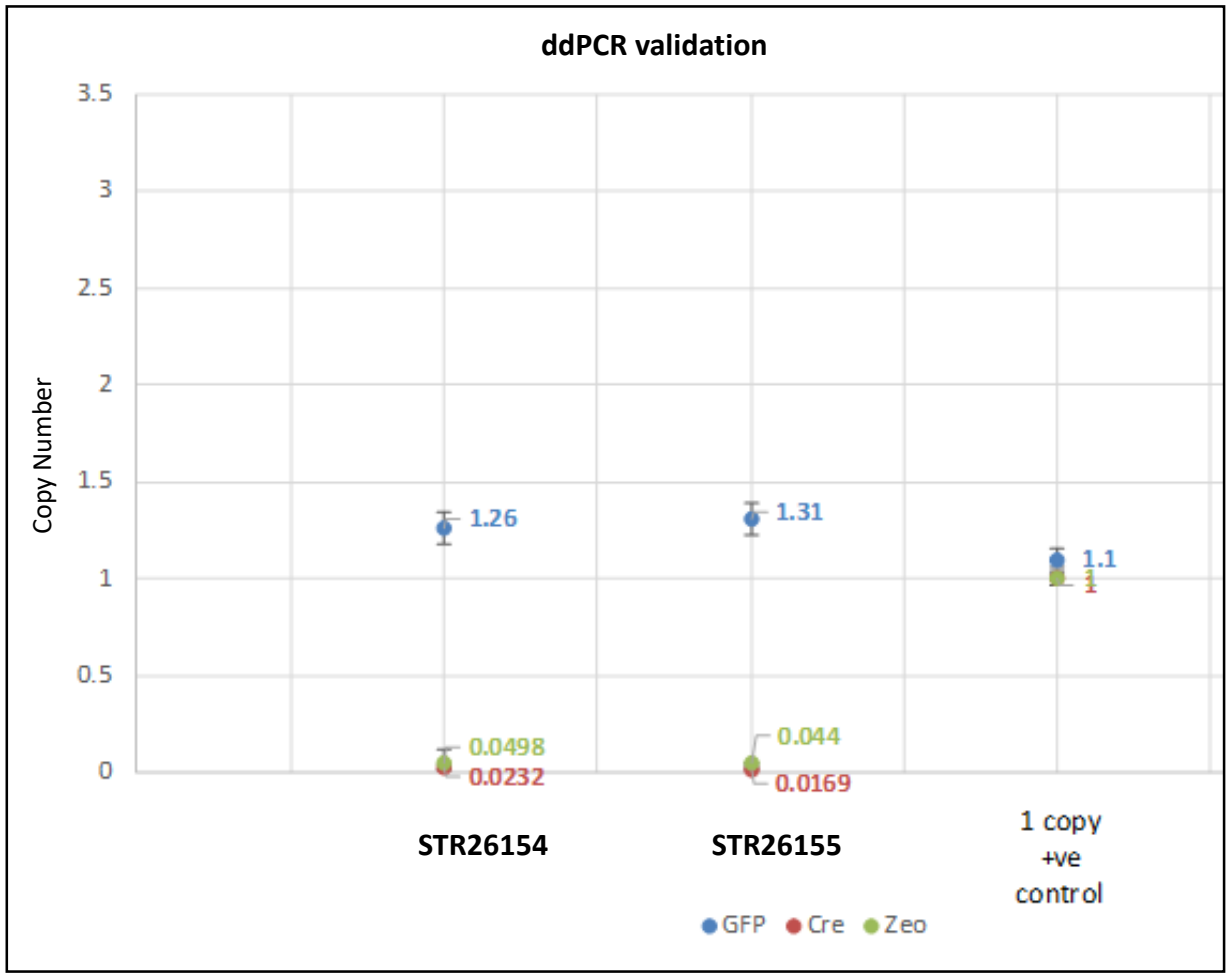


Figure C10: WGS read mapping to STR00010 reference genome at RS1 insertion site (annotations at top showing genes in blue and RS1 insertion site in green) for sequencing reads from subject strain STR26155 (middle) and recipient strain STR00012 (bottom). Lack of reads mapping across the RS1 insertion site confirms and validates insertion site.

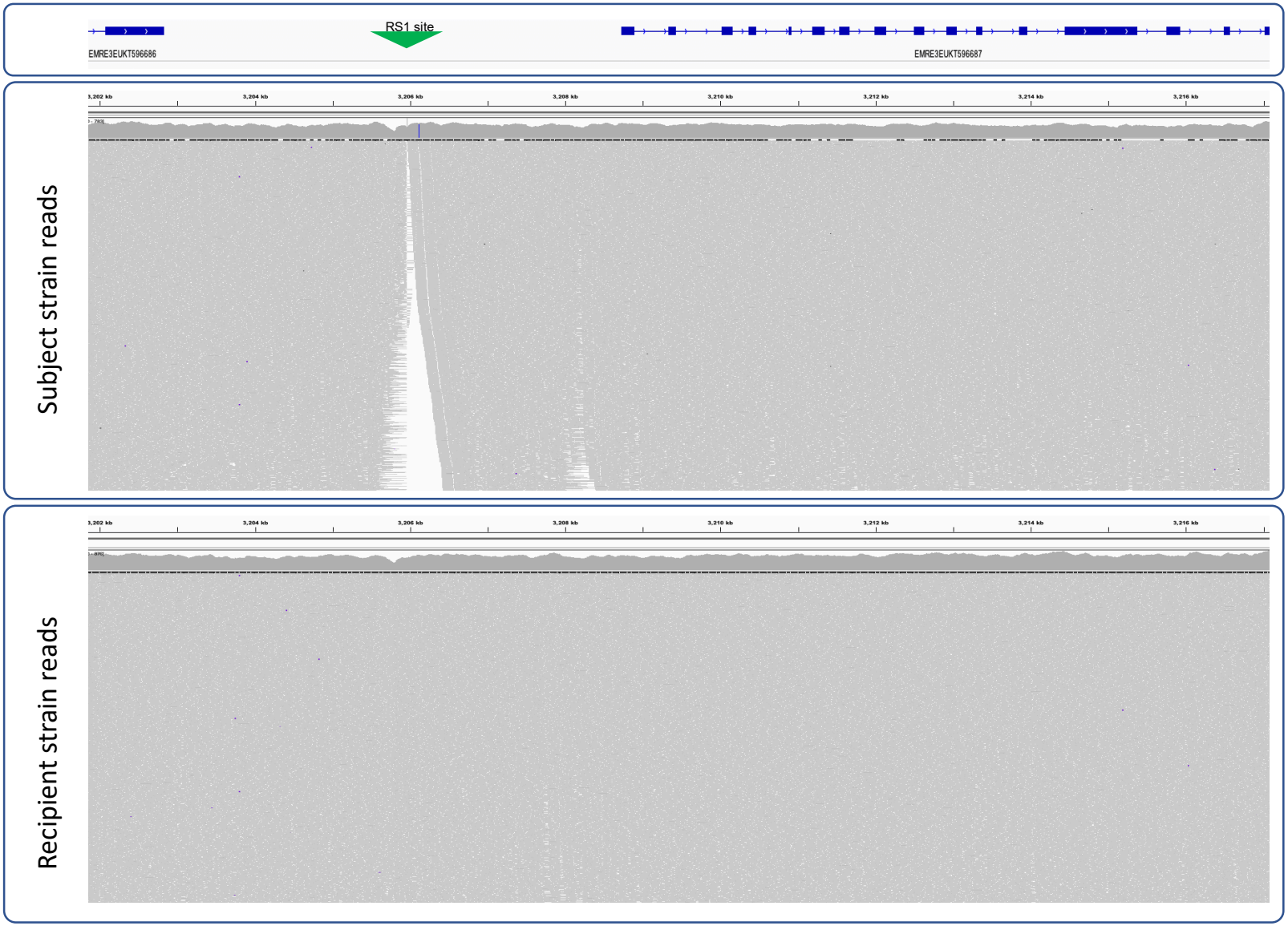


Figure C11: WGS read mapping to NAS14335. Linear representation with annotations provided at top panel, these are the same elements as in **Figure C2**. Middle panel depicts regions (also as in **Figure C2**) corresponding to vector backbone (in grey), insertion cassette (in blue) and material remaining in strain after Cre recombinase excision (in green). Bottom panel is read mapping showing the lack of reads pertaining to the vector backbone among the WGS data. As expected no sequences pertaining to the vector backbone are detected. Genetic elements used in intermediate construction do have mapped reads as these elements are present at their native genomic locations.

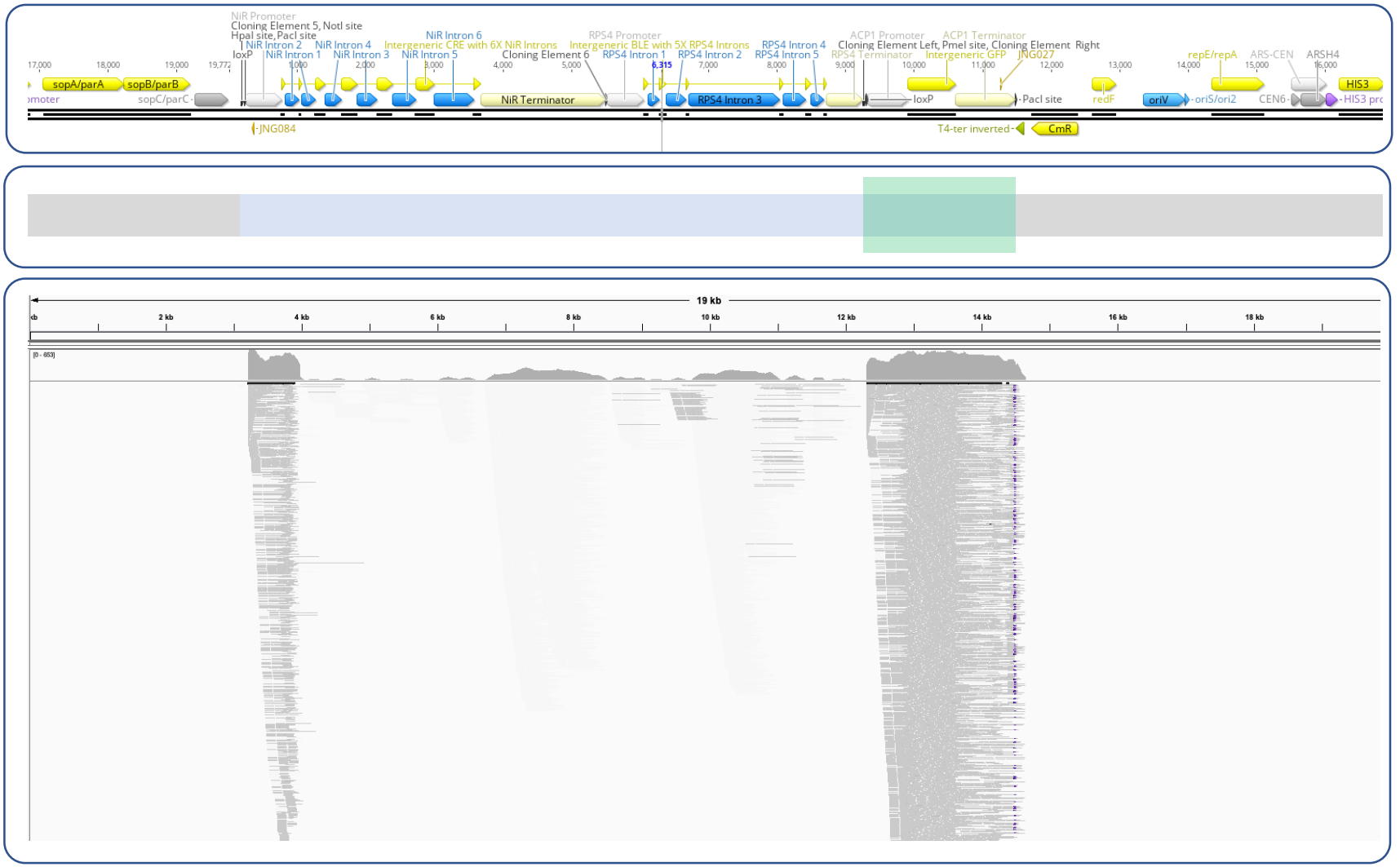


Figure C12: Read mapping to validated RS1 site containing GFP insert. Mapping recapitulates the expected sequence at the targeted locus. Close-ups are provided for nucleotide-level detail at insertion flanks.

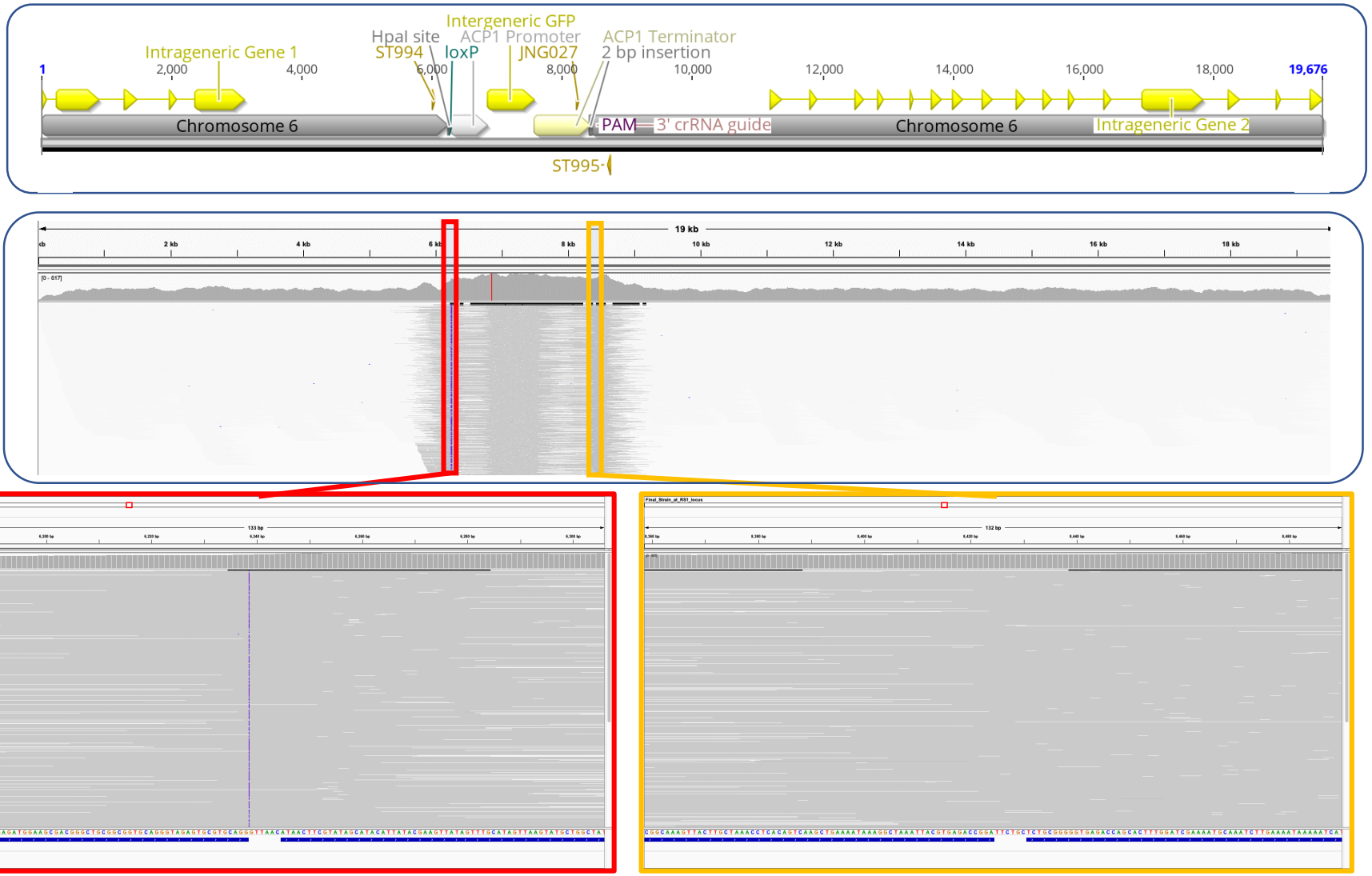


Figure F1: CAAF 2019 California Aquaculture Facility Registration

State of California - Natural Resources Agency
DEPARTMENT OF FISH AND WILDLIFE

GAVIN NEWSOM, Governor
CHARLTON H. BONHAM, Director

19 **Aquaculture Facility Registration** AQ
Valid 03/12/2019 to 12/31/2019

SYNTHETIC GENOMICS INC
250 W SCHRIMPF RD
CALIPATRIA, CA 92233

Aquaculture Facility:
- DAVID S. HANSELMAN/SYNTHETIC GENOMICS, INC, 250 WEST SCHRIMPF RD, CALIPATRIA, CA 92233

Types of Business Activity
- Live Hauler
- Processor
- Rears Aquatic Plants
- Research and Development

Registered Species
- Algae

Doc No: D-0025396063-4
Trans: 000000019311955
121503229

Outlet No: 310001-002
3/12/2019 9:33:08 AM

Item	Fee*
Aquaculture Reg. (Renew) 1st	$553.75
Prior Year Gross Sales: Less than $25,000	
Total:	**$553.75**

*Includes any applicable application fees, agent handling fees, and license buyer surcharge.

Gross Sales: A. Less than $25,000

STATE OF CALIFORNIA
DEPARTMENT OF FISH AND WILDLIFE
10/25/2018 10:56 AM
CDFW License and Revenue Branch
Outlet No: 310001-001
Clerk: MRAMADAN
Purchase Receipt
Trans #19693278
SYNTHETIC GENOMICS INC

Item Name	Amount
Aquaculture Fee Hold (Renew) 1st	553.75
Customer Total:	$553.75
CDFW Item Fees SubTotal:	$553.75
Transaction Total:	$553.75
CREDIT CARD	$553.75
Tender Amount:	$553.75
CHANGE:	($0.00)
Total Amount:	$553.75

I certify under penalty of perjury under the laws of the State of California tha
correct and that I meet the requirements for these licenses. I understand it is unlawful to make any false statement in this application or to use or possess a license obtained by fraud or deceit (Fish and Game Code §§1052(b) and 1054).

Name: _____ Title: _____ Signature: Signed copy maintained by SGI RA

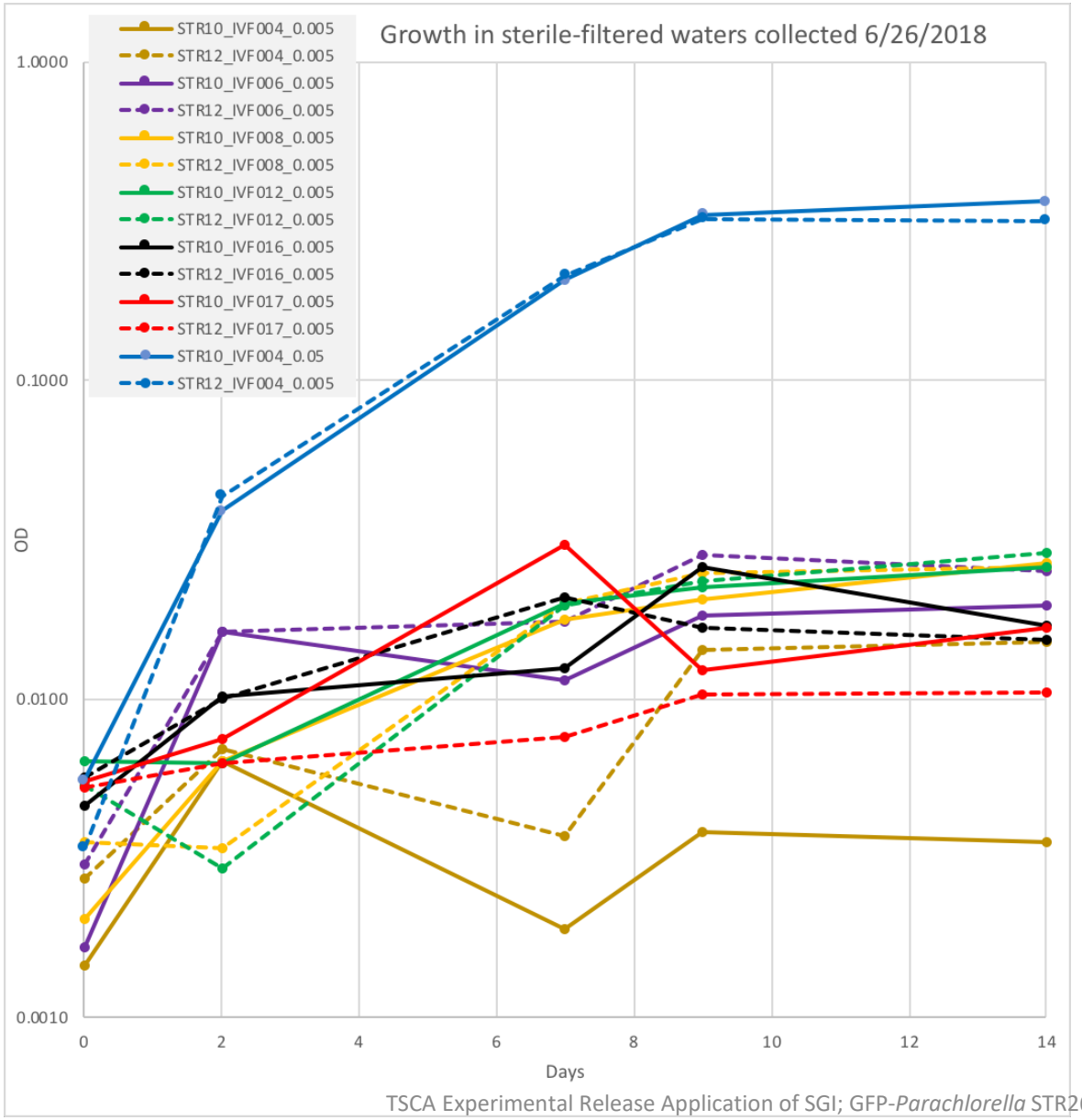
NOT VALID UNLESS SIGNED

Page 1 of 1

CA F&W Registered Algal Genera
Chlamydomonas spp.
Chlorella spp. (includes Parachlorella spp. in 2010)
Cyclotella spp.
Cylindrotheca spp.
Dunaliella spp.
Haematococcus spp.
Leptolyngbya spp.
Nannochloris spp.
Nannochloropsis spp.
Navicula spp.
Pseudochlorella spp.
Pseudoneochloris spp.
Scenedesmus spp.
Spirulina (Arthrospira) spp.
Synechococcus spp.
Synechocystis spp.
Tetraselmis spp.
Pavlova spp.
Schizochlamydella spp.
Isochrysis spp.
Porphyridium spp.
Tetrachlorella / Oocystis spp.
Klebsormidium spp.

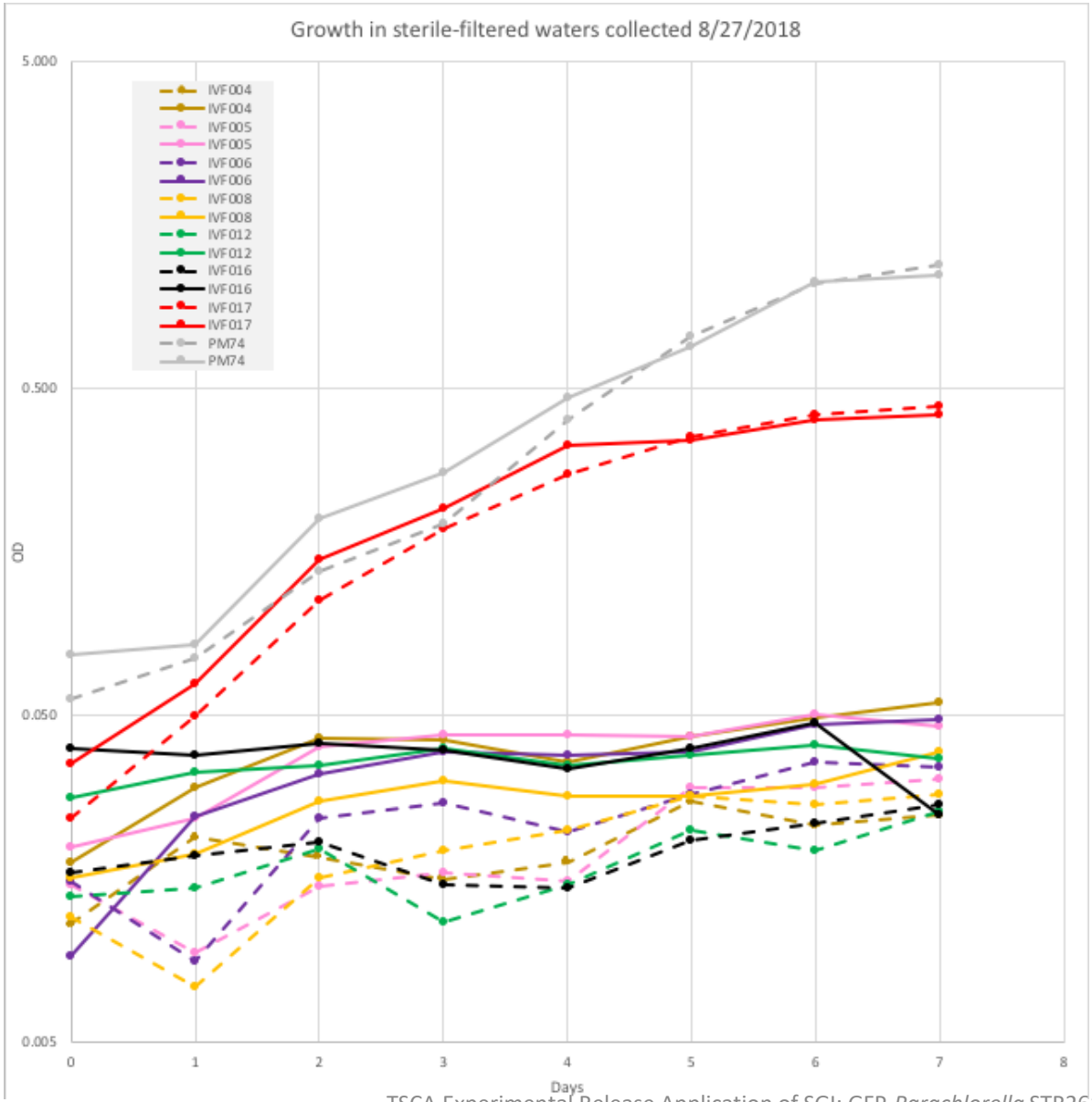
Figure F2: Aerial view of SGI's CAAF facility and local vicinity. The CAAF facility is bordered by the red rectangle. Environmental sampling stations are labeled and marked with red bulls-eyes.

Figure F3: Growth of wild-type *Parachlorella* STR00010 (solid lines) and recipient strain STR00012 (dashed line) in sterile-filtered waters. Sample water colored as in Figure F3. Only one inoculation density (0.005) shown for clarity. Sample naming convention: Strain_Site_Density.



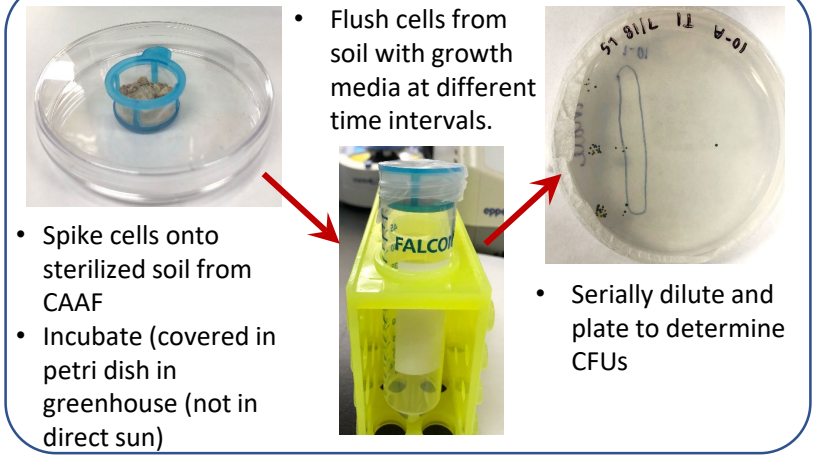
Sample Site ID	Description/Name
IVF001	CAAF Intake pond
IVF004	IID Input Canal
IVF005	Morton Bay
IVF006	Salton Sea Beach
IVF008	Salton Sea Obsidian Butte
IVF010	N MacDonald Rd
IVF012	Duck Pond
IVF016	IID Managed Marsh

Figure F4: Growth of recipient strain STR00012 (dashed line) and subject strain (solid line) in sterile-filtered waters. Sample water colored as in Figure F2. PM74 is included as a positive control. This is one of SGI's standard algal growth media which consists of artificial seawater and 10x added F/2 nutrients. Only one inoculation density (0.005) shown for clarity.



Sample Site ID	Description/Name
IVF001	CAAF Intake pond
IVF004	IID Input Canal
IVF005	Morton Bay
IVF006	Salton Sea Beach
IVF008	Salton Sea Obsidian Butte
IVF010	N MacDonald Rd
IVF012	Duck Pond
IVF016	IID Managed Marsh

Figure F5: Desiccation tolerance of recipient strain STR00012 and subject strain STR26155



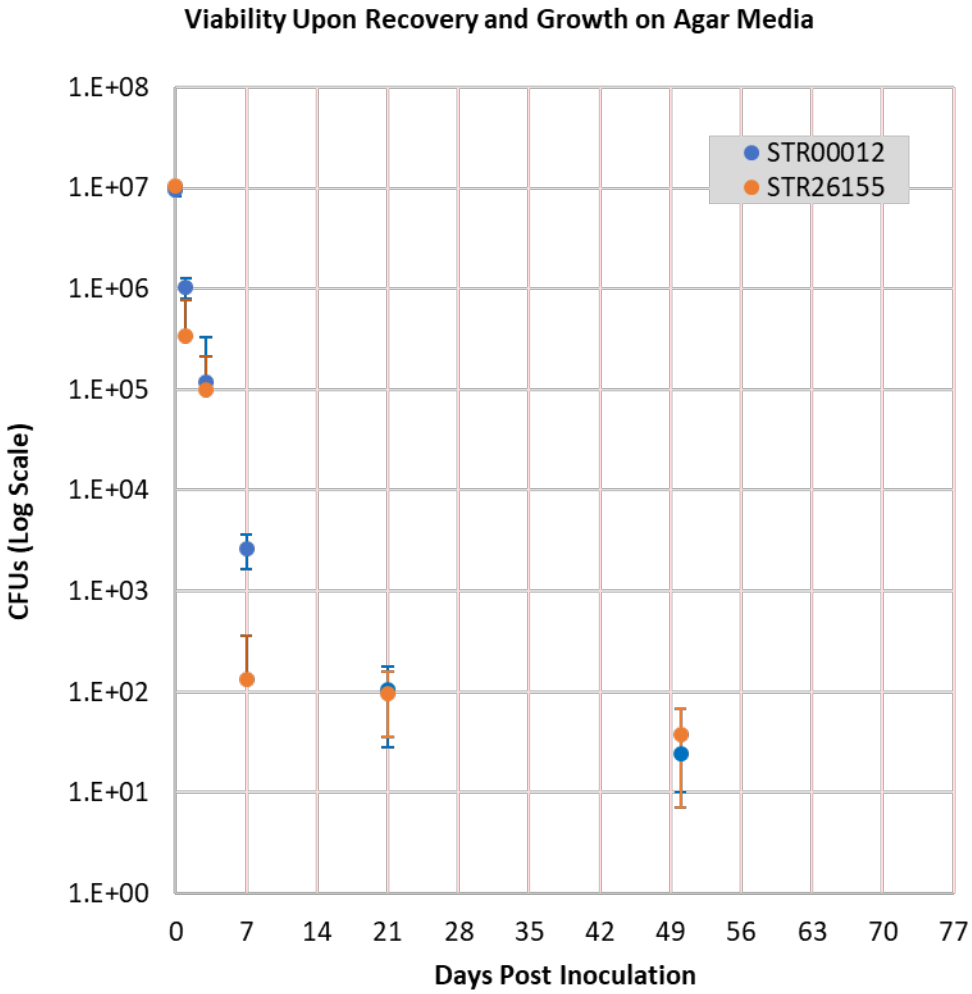
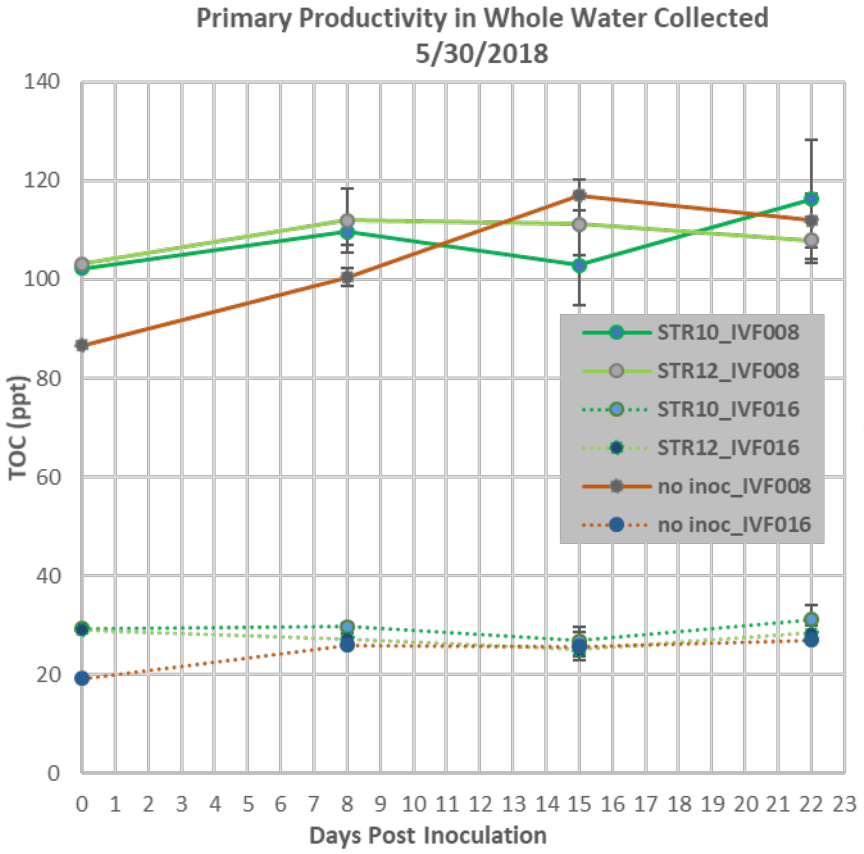
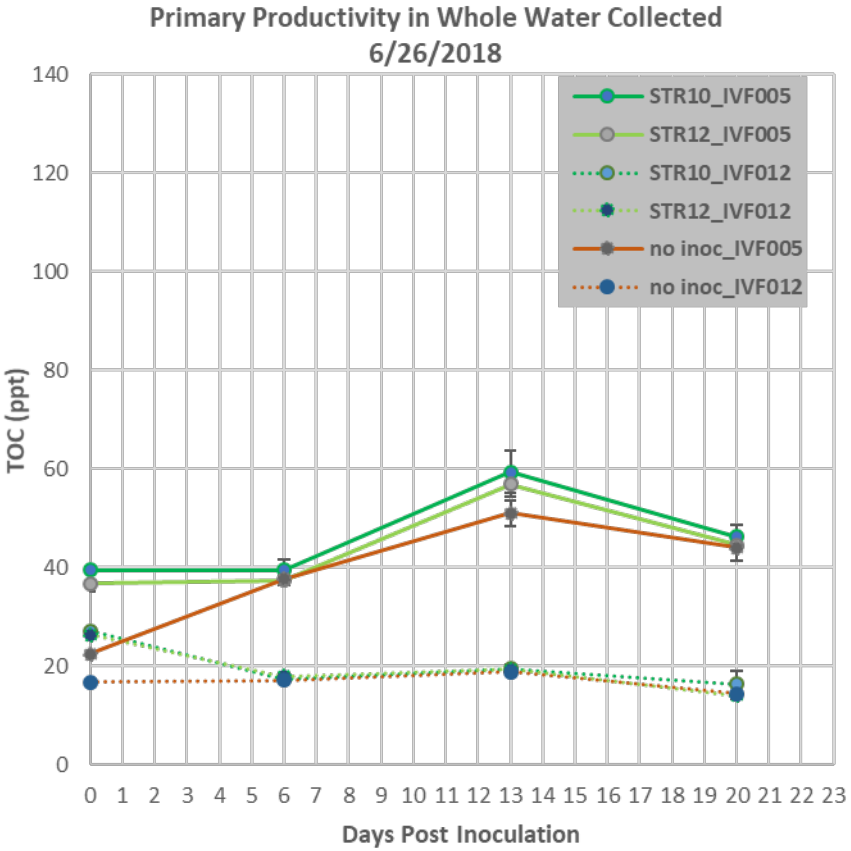


Figure F6: Whole-culture primary productivity (Total Organic Carbon, or TOC) measurements for waters spiked with *Parachlorella* STR00010 (dark green) recipient strain STR00012 (light green) and negative controls in unfiltered local waters. A) Waters collected from two sites, Salton sea (IVF008, solid lines) and IID managed marsh (IVF016, dotted lines) on 5/30/2018. B) Waters collected from two sites, Morton Bay (IVF005, solid lines) and duck/bass ponds (IVF012, dotted lines) on 6/26/2018.



IVF008 = Salton Sea, Obsidian Butte
IVF016 = Managed Marsh



IVF005 = Morton Bay
IVF012 = Duck / Bass Pond

Figure F7: Microbiome profiling data for invasion experiment using waters collected 6/26/2018 from Morton Bay (IVF005) and duck/bass ponds (IVF012). NMDS plots of microbiome profiling data show relative similarity of each microbiome based on clustering distance. All IVF005 samples (circled in blue) cluster discretely from IVF012 samples (circled in yellow). Clear progression over time is observed for both (light-grey dashed arrows) yet controls and treatment flasks cluster very closely indicating highly similar microbial communities.

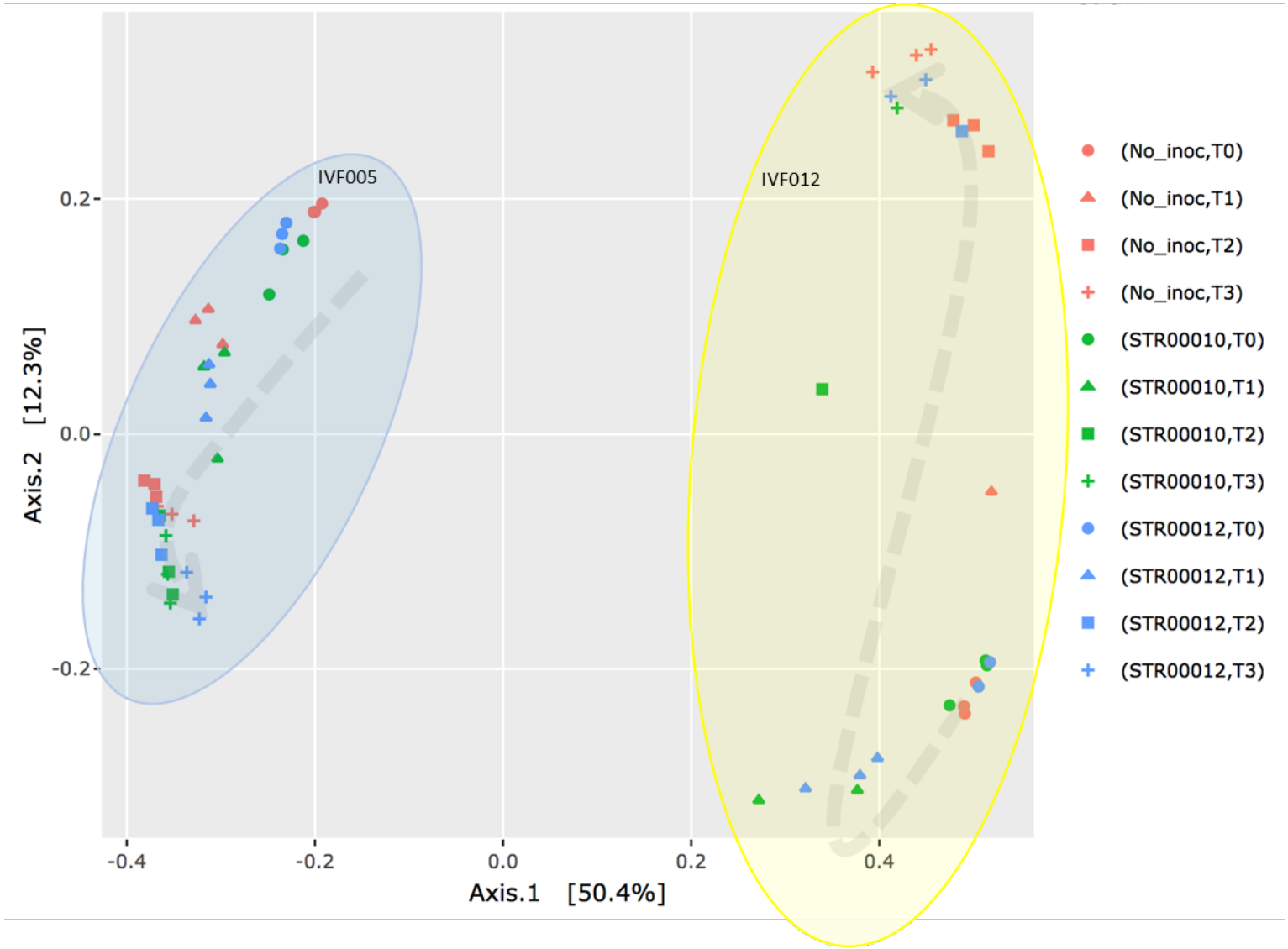


Figure F8: Whole-culture primary productivity (as Total Organic Carbon) measurements for waters spiked with recipient *Parachlorella* STR00012 (dark green) subject strain STR26155 (light green) and negative controls (blue) in unfiltered local waters. Waters collected from two sites, Salton Sea (IVF008, solid lines) and IID managed marsh (IVF016, dotted lines) on 8/27/2018.

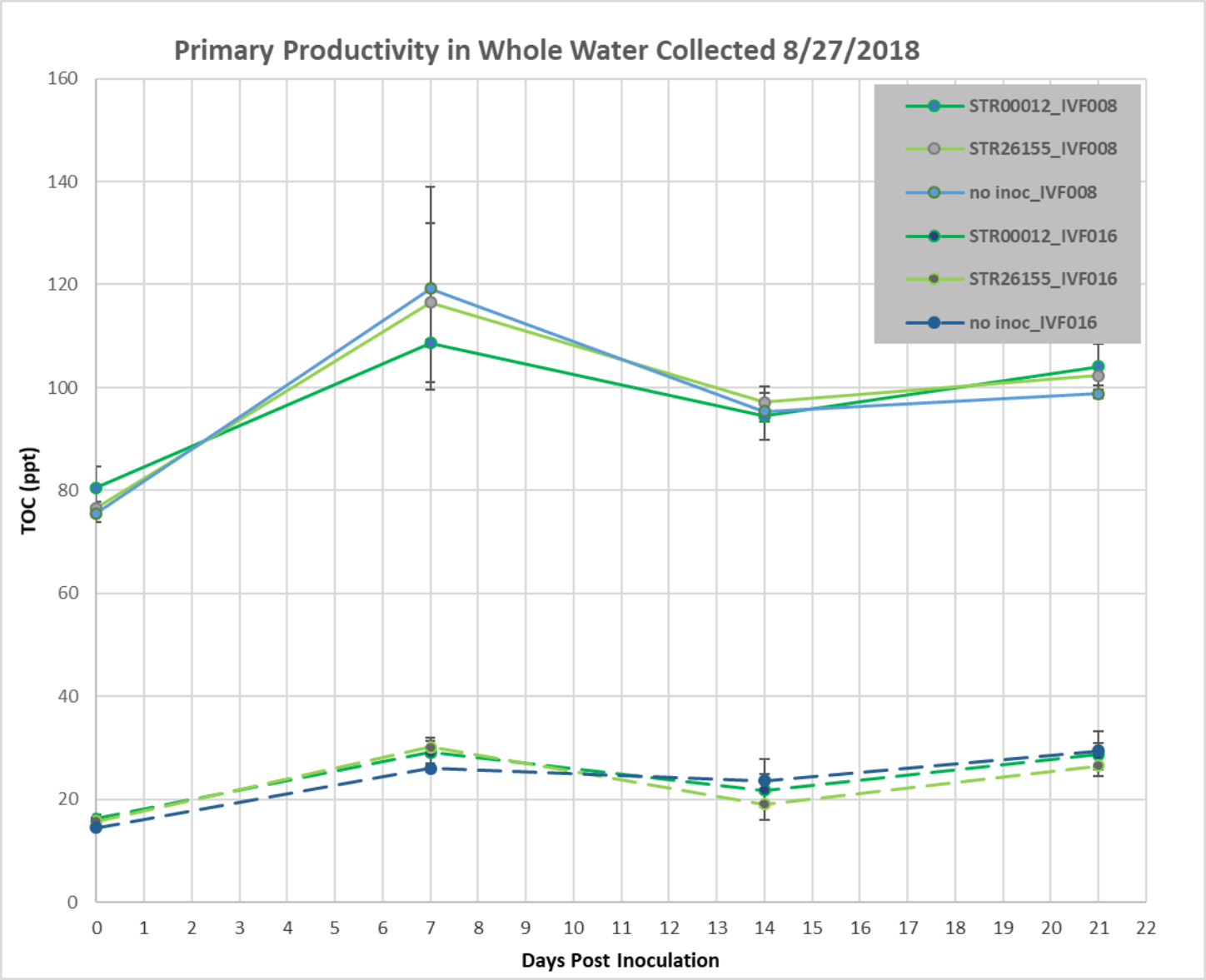


Figure F9: Whole-culture primary productivity (as Total Organic Carbon) measurements for waters spiked with subject *Parachlorella* STR26155 at three inoculation densities and negative controls. Inoculation densities are OD values (of the strains) high=0.05, medium=.005, low=.001. Waters were collected from two sites, Salton sea (IVF008, solid lines) and IID managed marsh (IVF016, dotted lines) on 10/4/2018.

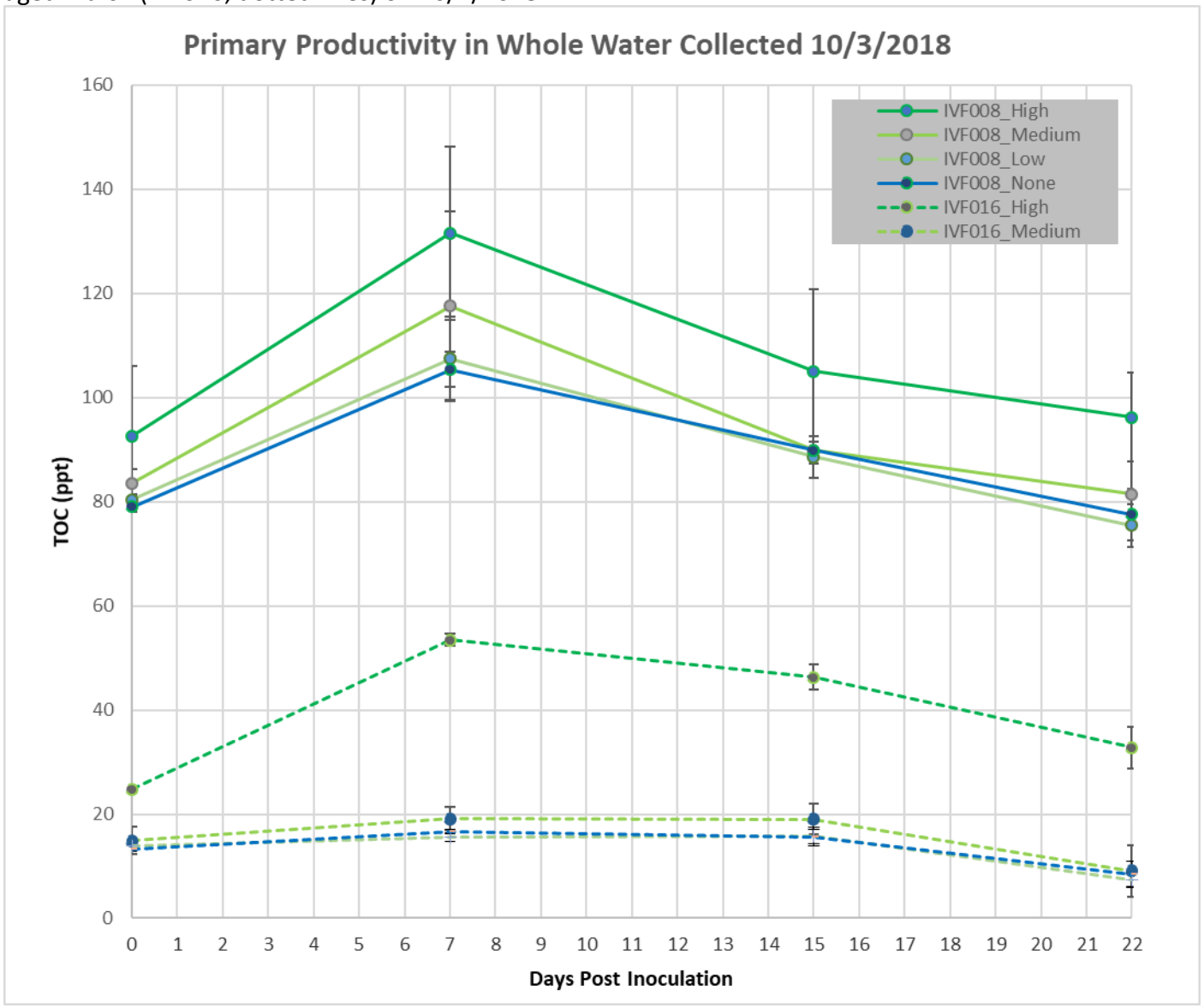


Figure F10: Microbiome profiling data for ALBOUT_0040. All IVF0016 samples (circled in blue) cluster discretely from IVF008 samples (circled in yellow). Clear progression over time is observed for both, yet controls and treatment flasks inoculated at a wide range of concentrations cluster very closely indicating highly similar microbial communities. Samples colored by inoculation level (L1= no inoculum, L2= 0.001 OD, L3= 0.005 OD, L4= 0.05 OD) and shape indicates timepoint (circle = T0, triangle = T1, square = T2, cross=T3).

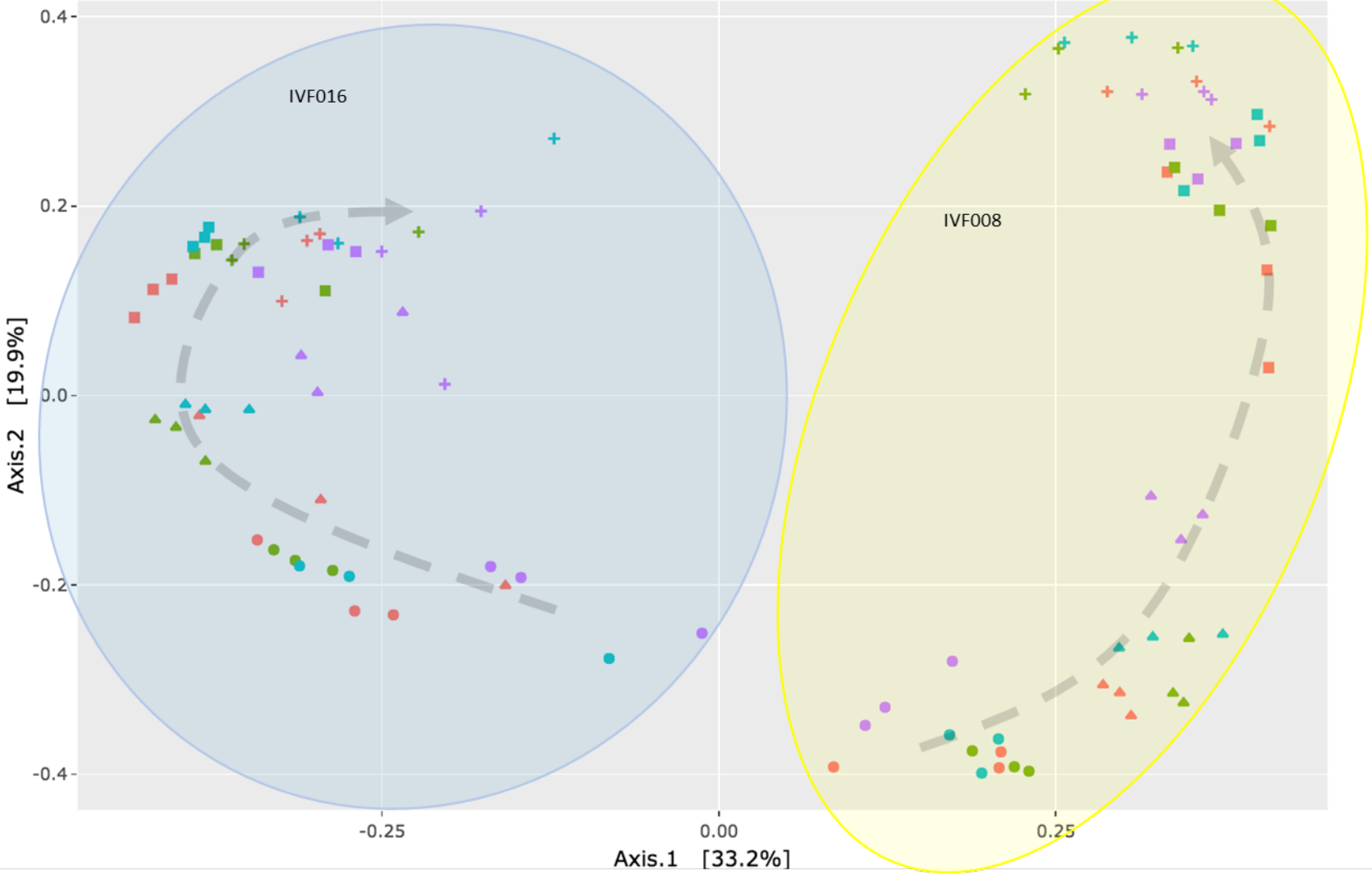


Figure G1: Satellite photo of CAAF facility (Feb-15). Structures and facilities of particular importance to this application are identified. The 0.1-acre ponds subject to this application and the greenhouse were built in 2019.

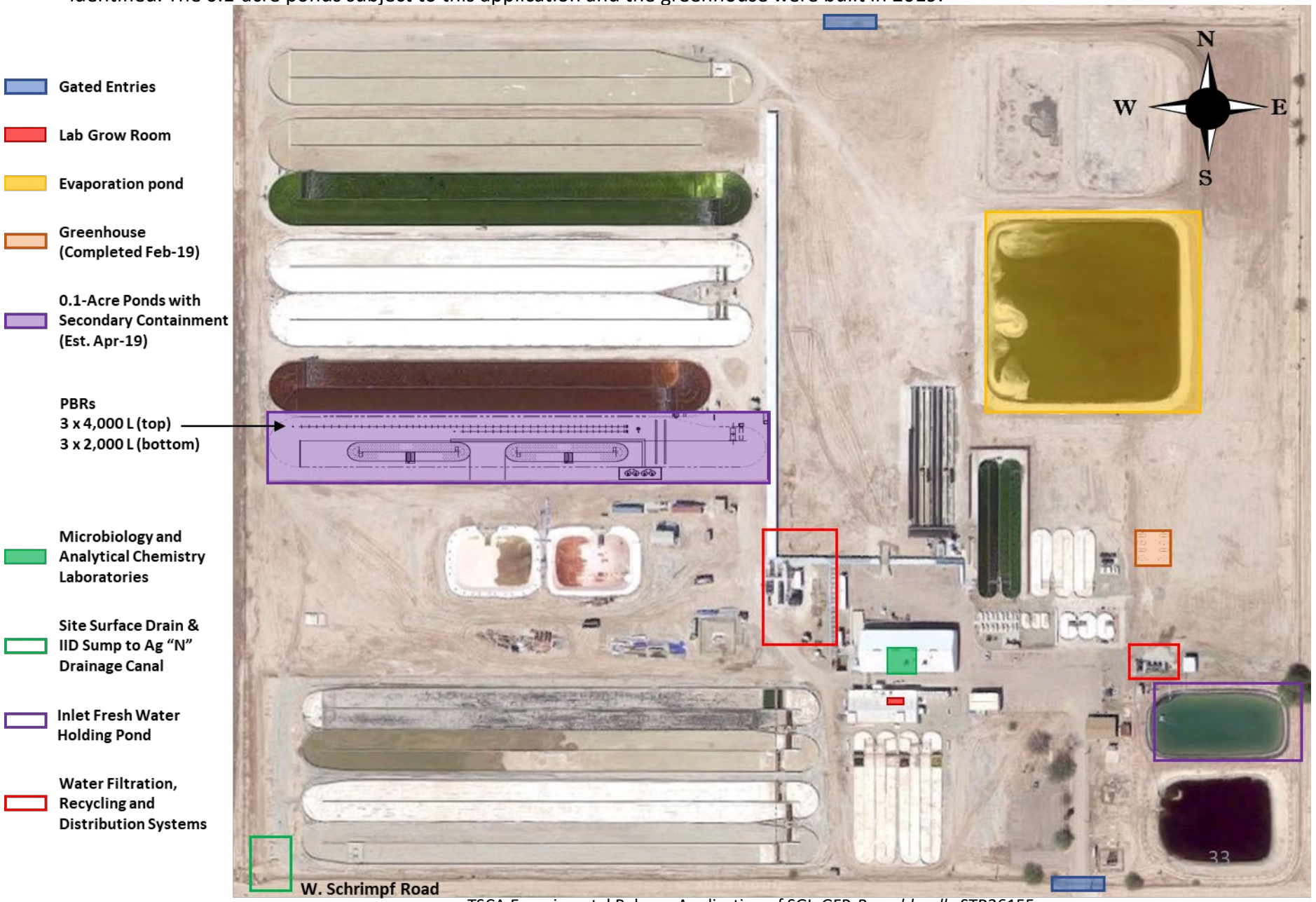


Figure G2: Summary of prevailing winds at CAAF site (direction denotes source direction of wind). (A) Frequency and direction daily average measurements for one year (2012) from Brawley CIMIS weather station. (B) Frequency and direction hourly averages from CAAF 2018 weather data for the months May-August (the anticipated months for outdoor ponds of the subject microorganism). Wind speed bins are in units of m/s.

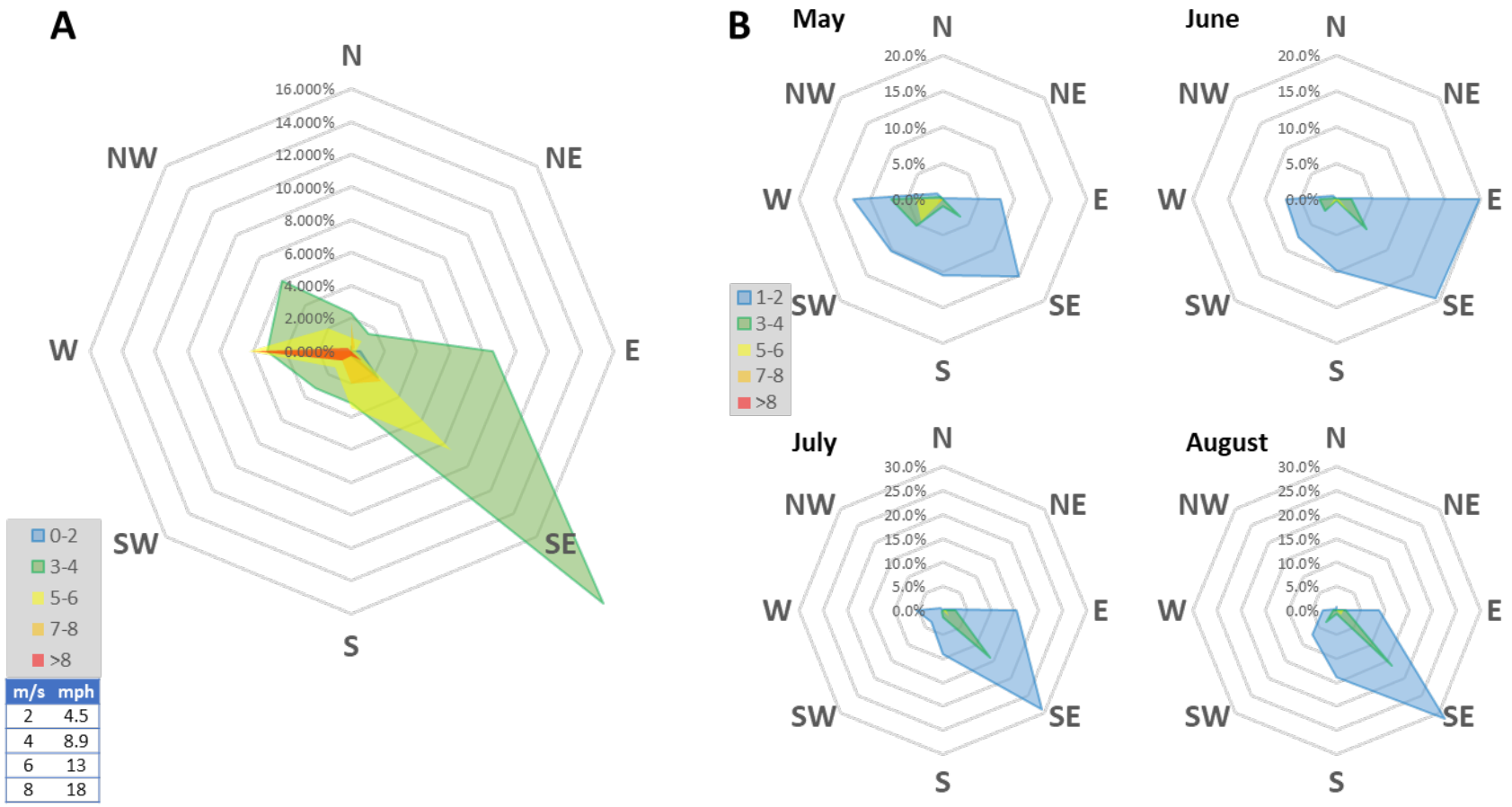


Figure G3: Process-waste evaporation-pond-liner engineering detail.

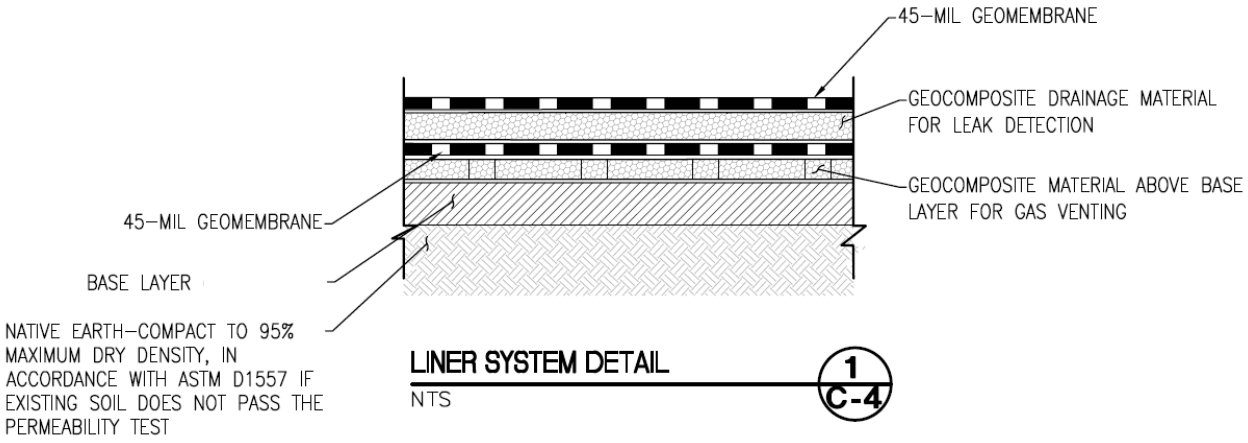


Figure H1: Geologic faults map of Salton Sea and areas surrounding CAAF facility and the Imperial Valley. Arrows indicate fault lines with highest potential impact to CAAF.

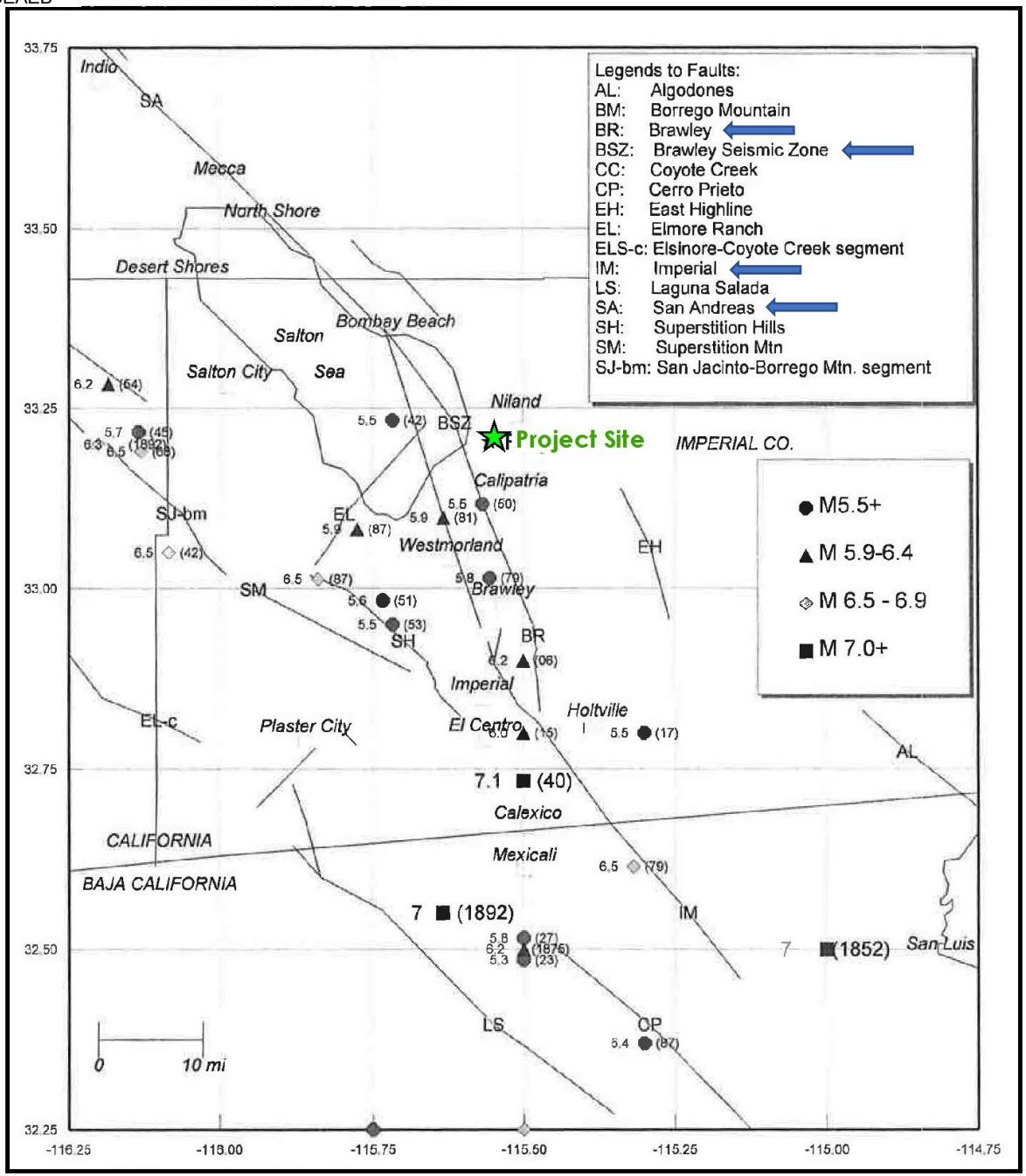


Figure H2: Soil categories, streams and wetlands on the CAAF site and adjacent areas.

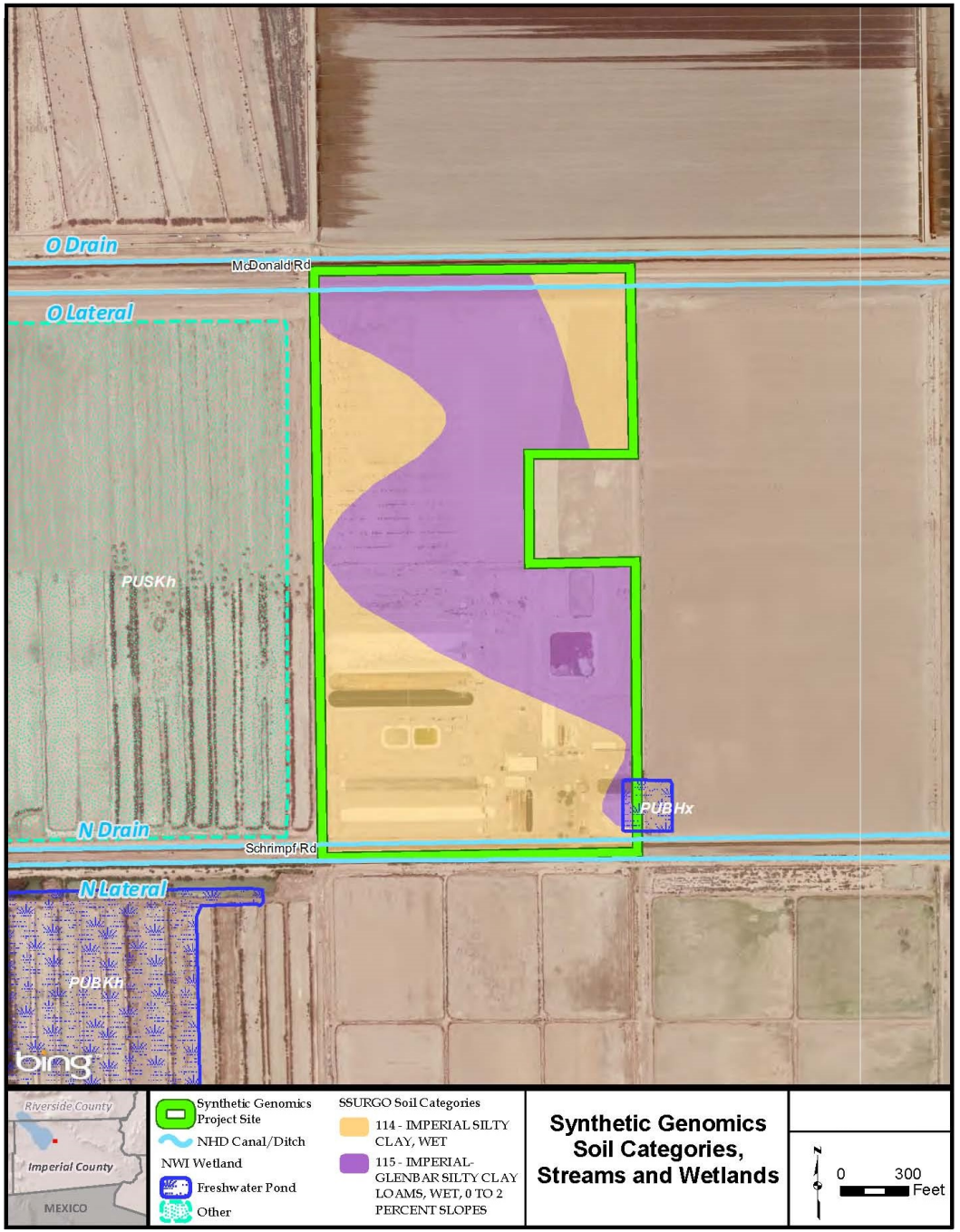


Figure H3: Topographic map of project area with neighboring municipalities, roads, water and geothermal features.

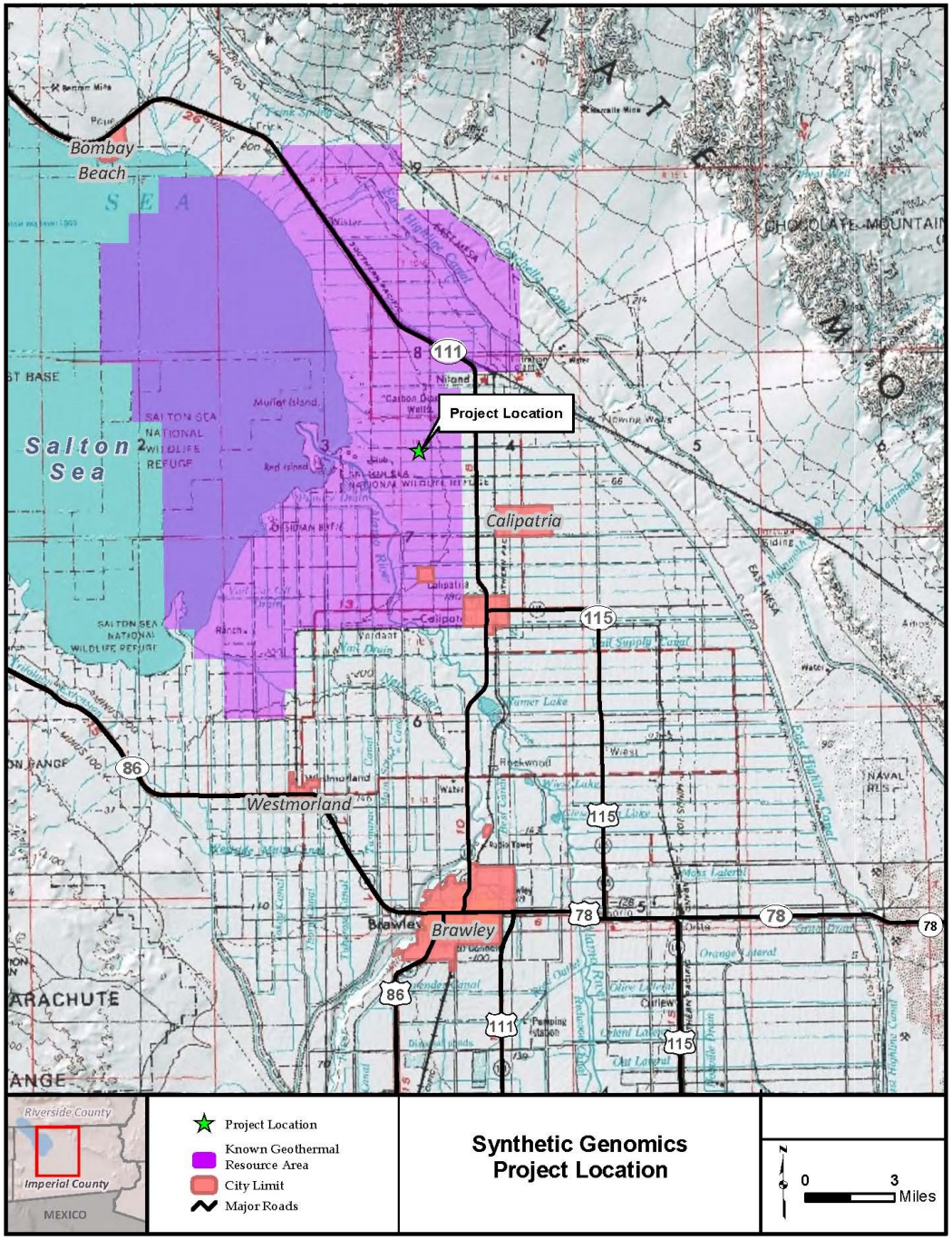
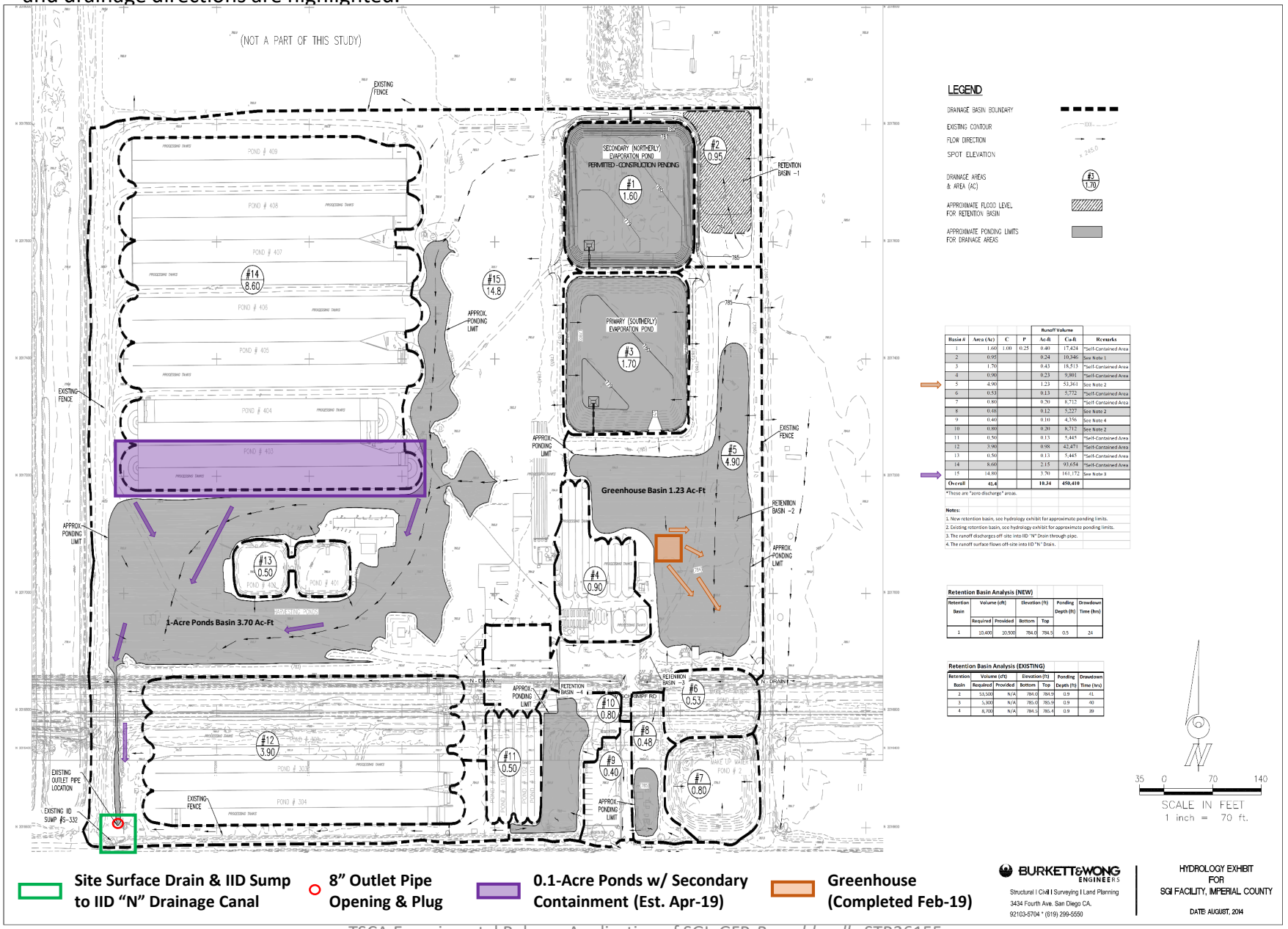


Figure H4: CAAF drainage study denoting retention basins and drainage. The 0.1-acre pond secondary containment area, greenhouse, and drainage directions are highlighted.



Basin #	Area (Ac)	C	P	Runoff Volume Ac-ft	Runoff Volume Cu-ft	Remarks
1	1.60	1.00	0.25	0.40	17,424	*Self-Contained Area
2	0.95			0.24	10,346	See Note 1
3	1.70			0.43	18,513	*Self-Contained Area
4	0.90			0.23	9,801	*Self-Contained Area
5	4.90			1.23	53,361	See Note 2
6	0.53			0.13	5,772	*Self-Contained Area
7	0.80			0.70	8,712	*Self Contained Area
8	0.48			0.12	5,227	See Note 2
9	0.40			0.10	4,356	See Note 4
10	0.80			0.20	8,712	See Note 2
11	0.50			0.13	5,445	*Self-Contained Area
12	3.90			0.98	42,471	*Self-Contained Area
13	0.50			0.13	5,445	*Self-Contained Area
14	8.60			2.15	93,654	*Self-Contained Area
15	14.80			3.70	161,172	See Note 3
Overall	41.4			10.34	450,410	

*These are "zero discharge" areas.

Notes:
1. New retention basin, see hydrology exhibit for approximate ponding limits.
2. Existing retention basin, see hydrology exhibit for approximate ponding limits.
3. The runoff discharges off-site into IID "N" Drain through pipe.
4. The runoff surface flows off-site into IID "N" Drain.

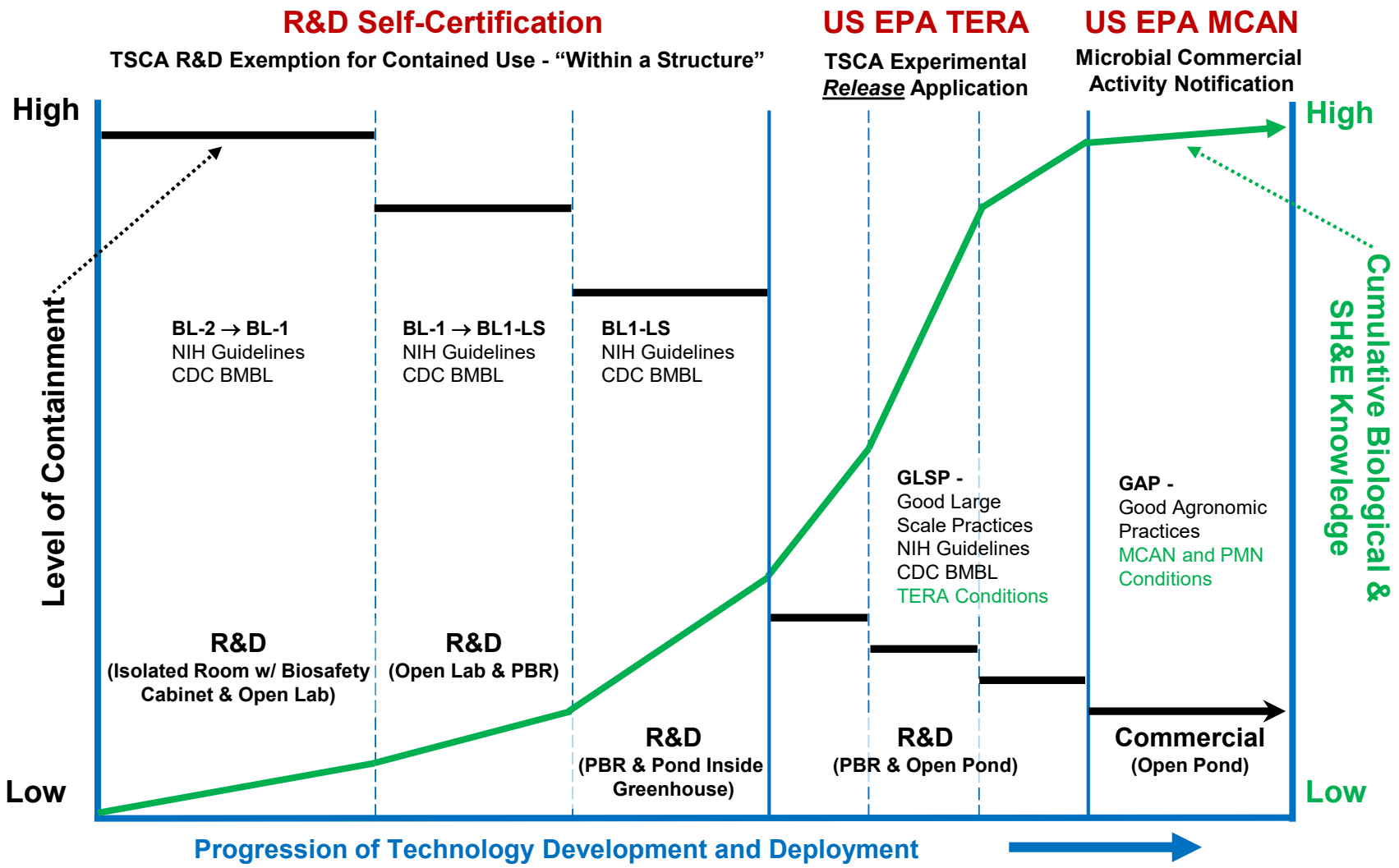
Retention Basin Analysis (NEW)

Retention Basin	Volume (cft)		Elevation (ft)		Ponding Depth (ft)	Drawdown Time (hrs)
	Required	Provided	Bottom	Top		
1	10,400	10,500	784.0	784.5	0.5	24

Retention Basin Analysis (EXISTING)

Retention Basin	Volume (cft)		Elevation (ft)		Ponding Depth (ft)	Drawdown Time (hrs)
	Required	Provided	Bottom	Top		
2	53,500	N/A	784.0	784.9	0.9	41
3	5,300	N/A	785.0	785.9	0.9	40
4	8,700	N/A	784.5	785.4	0.9	39

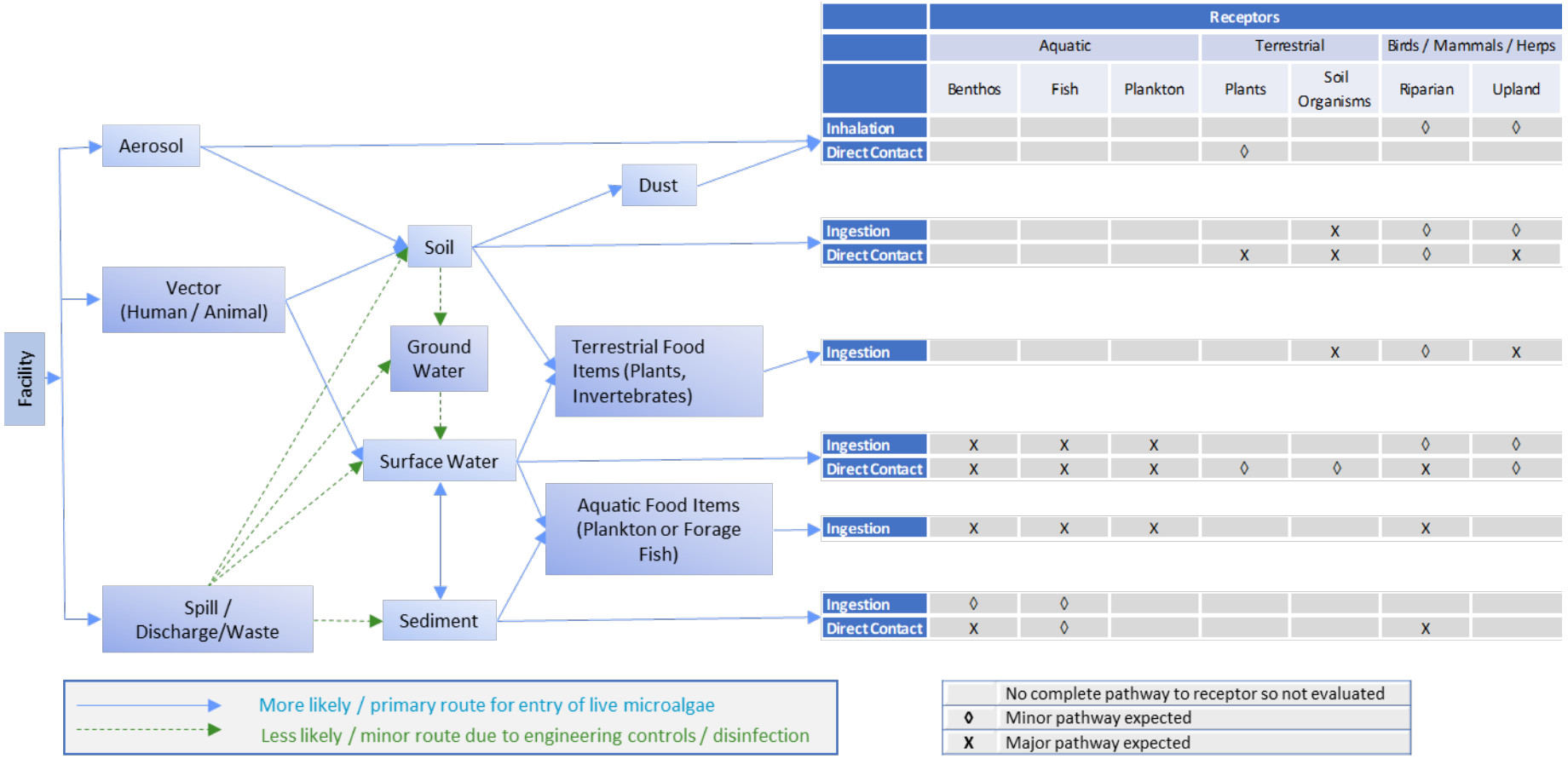
Figure I1: "Regulatory Learning Curve" for wild-type, classically-improved and engineered microorganisms: Physical containment (black horizontal bars) is a function of accumulated biological, safety, health and environmental knowledge (green curve)*.



PBR = Photo-bioreactor; BL-x (or BSL-x) = Biological Safety Level; LS = Large Scale; GLSP (or GILSP) = Good Industrial Large Scale Practices; GAP = USDA Good Agronomic Practices; BMBL = Biosafety in Microbiological and Biomedical Laboratories; TERA = TSCA Experimental Release Application; MCAN = Microbial Commercial Activity Notification; PMN: Pre-Manufacture Notice; NIH = US National Institutes of Health; CDC = US Centers for Disease Control; USDA: US Department of Agriculture; EPA: US Environmental Protection Agency.

* An earlier version of this figure was presented at a hearing held by the US National Academies of Science, Engineering and Medicine, Washington, DC, on 2-Jun-2016. http://nas-sites.org/biotech/files/2016/04/04-Hanselman.pdf

Figure I2: Conceptual transport model and potential exposure of ecological receptors to microalgae for the CAAF.



	Receptors						
	Aquatic			Terrestrial		Birds / Mammals / Herps	
	Benthos	Fish	Plankton	Plants	Soil Organisms	Riparian	Upland
Inhalation						◊	◊
Direct Contact				◊			
Ingestion					X	◊	◊
Direct Contact				X	X	◊	X
Ingestion					X	◊	X
Ingestion	X	X	X			◊	◊
Direct Contact	X	X	X	◊	◊	X	◊
Ingestion	X	X	X			X	
Ingestion	◊	◊					
Direct Contact	X	◊				X	

	No complete pathway to receptor so not evaluated
◊	Minor pathway expected
X	Major pathway expected

Figure J1: Identification of the facility environmental sampling locations. The approximate property boundary is in red. Secondary containment for enclosed photobioreactors and two 0.1 acre raceway ponds is identified in yellow. The approximate positions of algae trap ponds are in green.

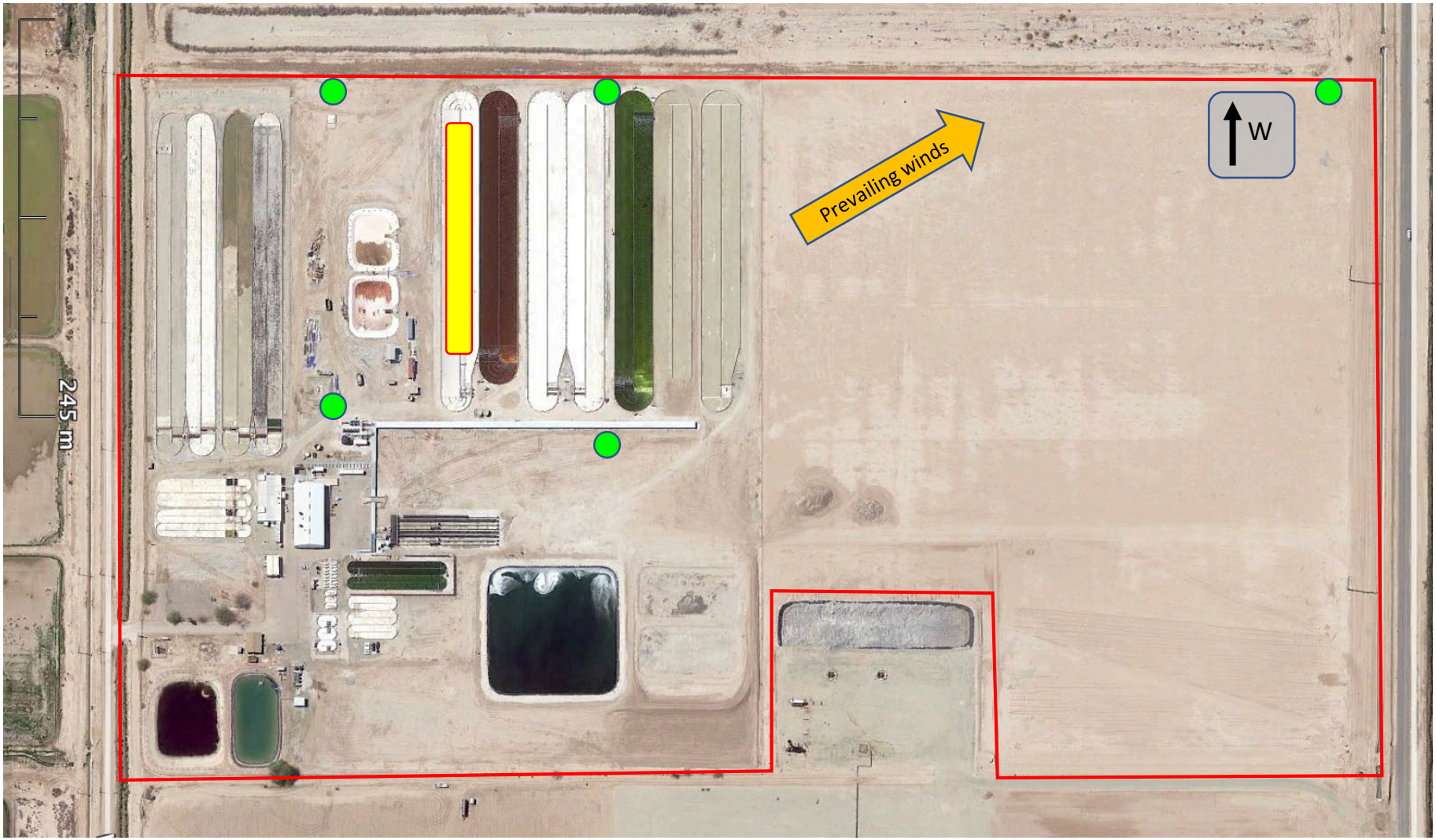


Figure J2: qPCR standard curve specific for the subject alga quantification.

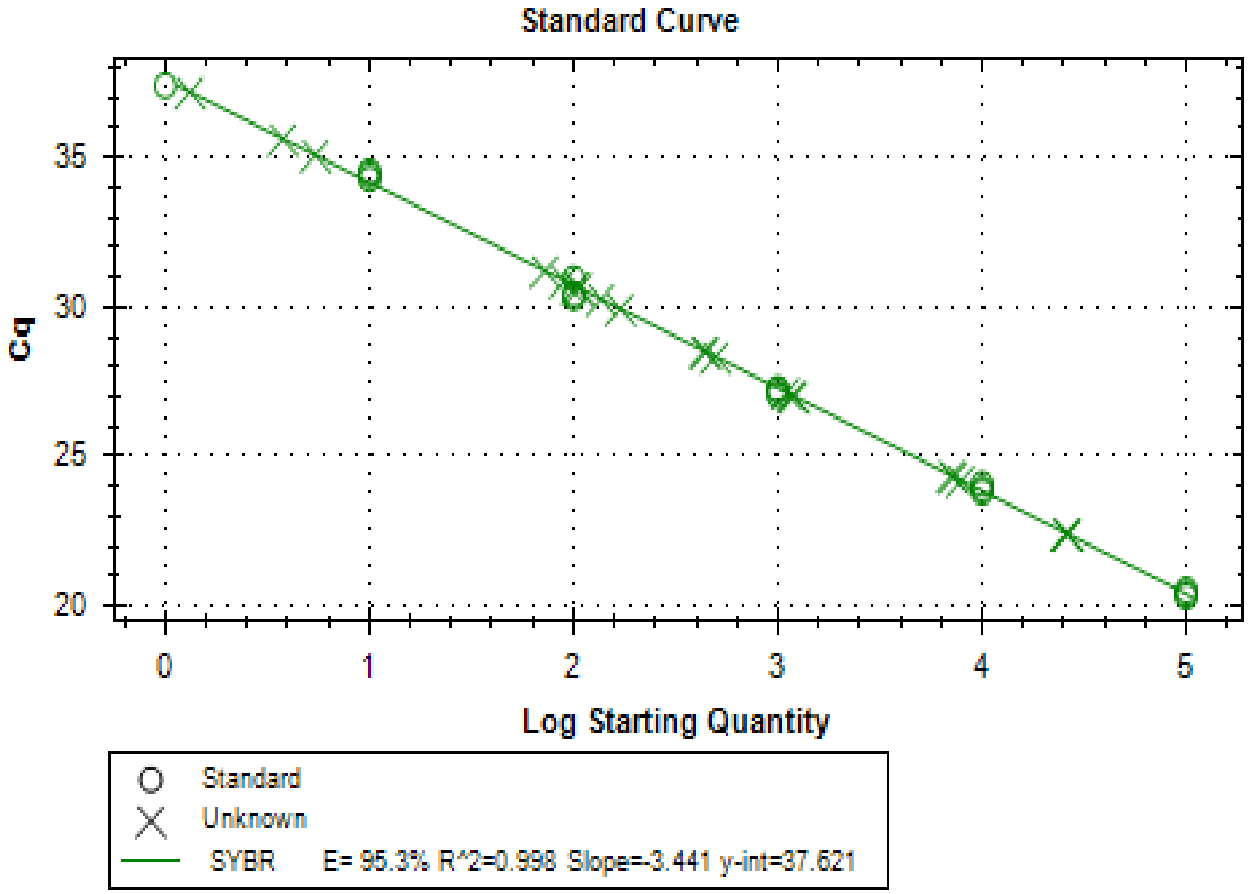
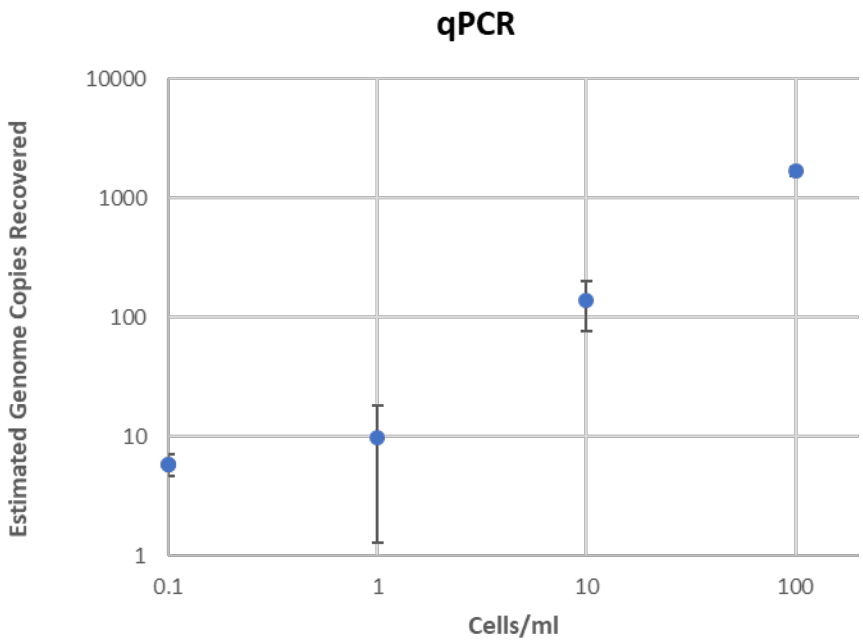


Figure J3: Detection and quantification of the subject alga in a complex environmental sample (Salton Sea water) by two orthogonal analytical approaches – qPCR and metabolic profiling.



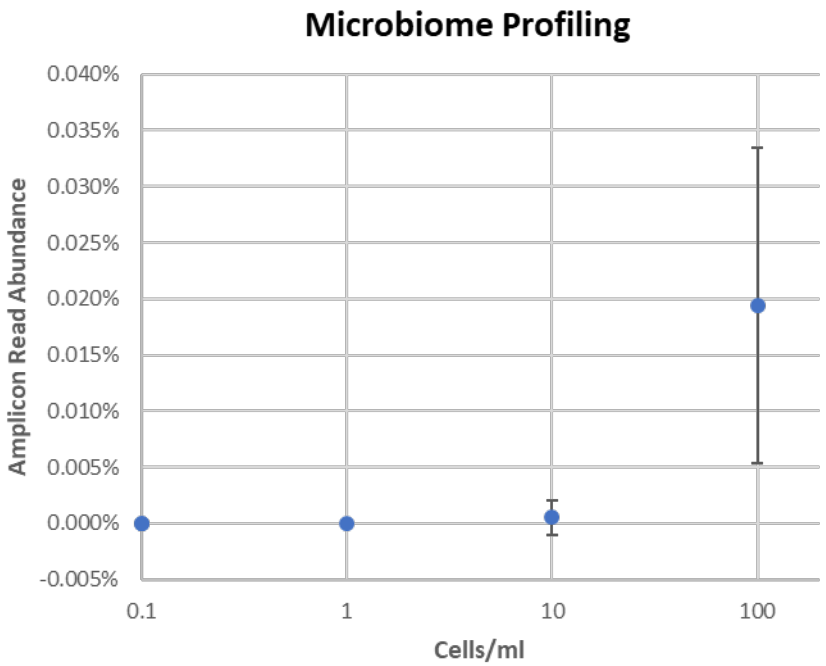


Table A1: Photophysiological characterization and comparison of recipient and subject strains. Errors are given in parentheses.

Strain#	FIRe (JTS-10)			^{14}C	Chlorophyll	
	F_V/F_M	σ_{PSII} (Å^2, @ 530 nm)	$1/\tau'_{Qa}$ (s^{-1})	P_{max} (nmol ^{14}C/ mg Chl/ hour)	(Chl a/TOC, %)	(Chl b/TOC, %)
STR00012	0.706 (0.010)	98 (2)	63 (2)	240 (1)	4 (0)	1 (0)
STR26155	0.708 (0.010)	101 (1)	64 (4)	238 (5)	4 (0)	1 (0)

JTS-10 parameters	Description
Fv/Fm	Maximum quantum yield of photochemistry in PSII, measured in a dark-adapted state (dimensionless). This parameter characterizes the efficiency of primary photosynthetic reactions.
σ_{PSII}	Functional absorption cross section of PSII (Å^2) in a dark-adapted state. The parameter is the product of the optical absorption cross section of PSII (i.e., the physical size of the PSII unit) and the quantum yield of photochemistry in PSII.
$1/\tau'_{Qa}$	Light saturated rate of electron transport on the acceptor side of photosystem II. This parameter indicates efficiency of linear photosynthetic electron transport

Table C1: Source information for intergeneric genes used in strain construction.

Gene	Source	CDS Modifications	Intron Present?
CRE	NCBI accession P06956	Codon optimized for *Parachlorella*	First six introns from *Parachlorella NiR* gene
BLE	NCBI accession Q6GKR3	Codon optimized for *Parachlorella*	First six introns from *Parachlorella RPS4* gene
GFP	Evrogen catalog #FP511	Codon optimized (by Evrogen, not specific to *Parachlorella*)	None

Table C2: Eight linear dsDNA fragments used in construction of plasmid NAS14355.

Fragment	Description	Source	Comments
1	Plasmid backbone (BAC)	Commercially available from Epicentre	-
2	Gibson overlap fragment from BAC to NiR-promoter	Synthesized by IDT	Includes loxP site, cloning linker element, and several restriction sites for cloning
3	NiR-promoter, CRE (with 6 NiR introns), NiR Term	Restriction fragment from plasmid	Cre synthesized by IDT, NiR promoter and terminator PCR amplified from recipient strain.
4	Gibson overlap fragment from NiR-terminator to RPS4 promoter	Synthesized by IDT	Includes cloning linker element and one restriction site
5	RPS4 Promoter, BLE (with 5 RPS4 introns, RPS4 Terminator	Restriction fragment from plasmid	BLE synthesized by IDT, RPS4 promoter and terminator PCR amplified from recipient strain. PCR and cloning resulted in 23bp deletion of terminator (relative to reference genome)
6	Gibson overlap fragment from RPS4-terminator to ACP1-promoter	Synthesized	Includes loxP site, cloning linker element, and several restriction sites for cloning
7	ACP1-promoter, TurboGFP, ACP1-terminator	Restriction fragment from plasmid	ACP1 promoter and terminator PCR amplified from recipient strain, cloned onto TurboGFP (from Evrogen)
8	Gibson overlap fragment from ACP1-terminator to BAC	Synthesized	Includes two restriction sites

Table C3: Details of the genetic elements on plasmid NAS14335. Blank entries are either restriction enzyme sites or fully synthetic sequences, none of which are organism-specific. Intergeneric elements present in the final construct have darkened borders.

Genetic Element	Type	Source	Source Detail	Position Start	Position End	Length (bp)	Present in Final Strain?
PacI site	Cloning Element	Intergeneric		175	182	8	No
HpaI site	Cloning Element	Intergeneric		183	188	6	Yes
loxP	lox site	Intergeneric	P1 bacteriophage	189	222	34	Yes
NotI site	Cloning Element	Intergeneric		223	230	8	No
Cloning Linker 5	Cloning Element	Synthetic		231	255	25	No
NiR Promoter	Promoter	Intrageneric	*Parachlorella*	256	767	512	No
JNG084	Primer binding site	NA	NA	347	365	19	No
CRE with 6X NiR Introns	CDS	Intergeneric	P1 bacteriophage	768	3676	1056	No
NiR Intron 1	Intron	Intrageneric	*Parachlorella*	819	1027	209	No
NiR Intron 2	Intron	Intrageneric	*Parachlorella*	1062	1258	197	No
NiR Intron 3	Intron	Intrageneric	*Parachlorella*	1406	1643	238	No
NiR Intron 4	Intron	Intrageneric	*Parachlorella*	1873	2162	290	No
NiR Intron 5	Intron	Intrageneric	*Parachlorella*	2383	2724	342	No
NiR Intron 6	Intron	Intrageneric	*Parachlorella*	2997	3573	577	No
NiR Terminator	Terminator	Intrageneric	*Parachlorella*	3677	5490	1814	No
Cloning Linker 6	Cloning Element	Synthetic		5491	5515	25	No
AsiS site	Cloning Element	Intergeneric		5516	5523	8	No
RPS4 Promoter	Promoter	Intrageneric	*Parachlorella*	5524	6054	531	No
BLE with 5X RPS4 Introns	CDS	Intergeneric	*S. hindustanus*	6055	8721	375	No
RPS4 Intron 1	Intron	Intrageneric	*Parachlorella*	6122	6277	156	No
RPS4 Intron 2	Intron	Intrageneric	*Parachlorella*	6379	6670	292	No
RPS4 Intron 3	Intron	Intrageneric	*Parachlorella*	6711	8032	1322	No
RPS4 Intron 4	Intron	Intrageneric	*Parachlorella*	8085	8410	326	No
RPS4 Intron 5	Intron	Intrageneric	*Parachlorella*	8488	8683	196	No
RPS4 Terminator	Terminator	Intrageneric	*Parachlorella*	8722	9244	523	No
Cloning Linker Left	Cloning Element	Synthetic		9245	9269	25	No
PmeI site	Cloning Element	Intergeneric		9266	9273	8	No
Cloning Linker Right	Cloning Element	Synthetic		9270	9294	25	No
loxP	lox site	Intergeneric	P1 bacteriophage	9295	9328	34	No
ACP1 Promoter	Promoter	Intrageneric	*Parachlorella*	9329	9900	572	Yes
GFP	CDS	Intergeneric	Calanoid copepod	9901	10602	702	Yes
ACP1 Terminator	Terminator	Intrageneric	*Parachlorella*	10603	11471	869	Yes
JNG027	Primer binding site	NA	NA	11257	11278	22	Yes
PacI site	Cloning Element	Intergeneric		11478	11485	8	No
T4-ter inverted	T4-ter inverted	Plasmid	Epicentre pCC1BAC	11486	11596	111	No
CmR	CDS	Plasmid	Epicentre pCC1BAC	11717	12376	660	No
redF	CDS	Plasmid	Epicentre pCC1BAC	12595	12942	348	No
oriV	rep_origin	Plasmid	Epicentre pCC1BAC	13337	13954	618	No
oriS/ori2	rep_origin	Plasmid	Epicentre pCC1BAC	13955	14009	55	No
repE/repA	CDS	Plasmid	Epicentre pCC1BAC	14338	15093	756	No
CEN6	misc_feature	Plasmid	Epicentre pCC1BAC	15508	15624	117	No
ARS-CEN (template = pARS-VN)	old_sequence	Plasmid	Epicentre pCC1BAC	15508	16012	505	No
ARSH4	misc_feature	Plasmid	Epicentre pCC1BAC	15638	16012	375	No
HIS3 promoter (Struhl 1982)	promoter uukaryotic	Plasmid	Epicentre pCC1BAC	16013	16172	160	No
HIS3	CDS	Plasmid	Epicentre pCC1BAC	16199	16861	663	No
sopA/parA	CDS	Plasmid	Epicentre pCC1BAC	17060	18226	1167	No
sopB/parB	CDS	Plasmid	Epicentre pCC1BAC	18226	19197	972	No
sopC/parC	misc_feature	Plasmid	Epicentre pCC1BAC	19270	19743	474	No

Table C4: Commercial sources for DNA and other elements used in strain construction.

Item	Source	Description	Catalogue Number
BAC backbone	Epicentre	CopyControl™ pCC1BAC™ Vector	CBAC311B
TURBO GFP	Evrogen	TurboGFP, an improved GFP variant originally sourced from a copepod.	FP515
CAS9-3NLS	IDT	CAS9 V3*: Recombinant *S. pyogenes* Cas9 nuclease, purified from an *E. coli* strain expressing the nuclease. Contains nuclear localization sequence (NLS) and C-terminal 6-His tag	1081059 for current version
Alt-R® CRISPR-Cas9 crRNA, 2 nmol	IDT	Must be used with tracrRNA to form a functional gRNA duplex. Suitable for most applications. Contain chemical modifications to protect from degradation by cellular RNases.	Custom sequence: GAGTGCGTGCAG GAGTTCTG
Alt-R® CRISPR-Cas9 tracrRNA, 100 nmol	IDT	Universal 67mer tracrRNA that contains proprietary chemical modifications conferring increased nuclease resistance. Hybridizes to crRNA to activate the Cas9 enzyme	1072534

Table C5: Details of introduced genetic elements on chromosome 6 (and associated annotations).

Genetic Element	Type	Source	Source Detail	Length (bp)	Position Start	Position End
Chromosome 6	misc. feature	intrageneric	endogenous genome		start	635
ST994	oligo binding site for genotyping		endogenous genome	25	398	422
HpaI site	cloning Element	intergeneric		6	636	641
loxP	lox site	intergeneric	P1 bacteriophage	34	642	675
ACP1 Promoter	promoter	intrageneric	*Parachlorella*	572	676	1247
GFP	CDS	intergeneric	Calanoid copepod	702	1248	1949
ACP1 Terminator	terminator	intrageneric	*Parachlorella*	869	1950	2818
2 bp insertion	misc. feature			2	2826	2827
3' crRNA guide	crRNA guide region		endogenous genome	3	2829	2831
PAM site	PAM site		endogenous genome	3	2832	2834
ST995	oligo binding site for genotyping		endogenous genome	29	3108	3136
Chromosome 6	misc. feature	intrageneric	endogenous genome		2828	end

Table E1: Qualitative ecological assessment of GFP-engineered microalgae

Hazard Assessment of Microalgae (Engineered)		
Receptors	**Toxicity/Pathogenicity**	**Basis**
Terrestrial Plants	IV	No record of adverse impacts of the proposed genera of microalgae to terrestrial plants
Terrestrial Animals	IV	No record of adverse impacts of the proposed genera of microalgae to terrestrial animals .
Aquatic Plants	III/IV	No record of toxicity or pathogenicity of the proposed genera of microalgae to aquatic plants; there is some potential for population effects related to competition/biogeochemistry
Aquatic Wildlife	III/IV	No record of toxicity or pathogenicity of the proposed genera of microalgae to aquatic wildlife; there is some potential for population effects related to competition/biogeochemistry
I. Toxicity/Pathogenicity: well-established potential for acute toxicity or pathogenicity that is likely to cause significant population-level impairment and/or impact ecologically or economically valuable species.		
II. Toxicity/Pathogenicity: Documented or suspected potential for toxic effect, including neurotoxins or established infection by species/strain; widespread effects; direct or indirect effects on Threatened or Endangered species.		
III. Toxicity/Pathogenicity: Limited potential for localized, population-level effects or limited evidence of isolated infection of a host species within a given genus. Minimal toxicity predicted from environmental exposure.		
IV. Toxicity/Pathogenicity: Minimal toxicity predicted from environmental exposure. History of environmental presence of the species indicates no direct toxic effects from high rates of exposure (e.g. as food).		

Table F1: Description and location of sampling stations in the vicinity of the CAAF.

Sample Site ID	Description/Name	Latitude	Longitude
IVF001	CAAF Intake pond	33.174910	-115.638490
IVF004	IID Input Canal	33.205433	-115.557765
IVF005	Morton Bay	33.201680	-115.597140
IVF006	Salton Sea Beach	33.161970	-115.647030
IVF008	Salton Sea Obsidian Butte	33.175250	-115.638483
IVF010	N MacDonald Rd	33.206344	-115.565372
IVF012	Duck Pond	33.197521	-115.568531
IVF016	IID Managed Marsh	33.205902	-115.527130
IVF017	Alamo River	33.199112	-115.597050

Table G1: Sampling frequency and measurement type.

Measurement Type	Frequency	Sampling Location
OD_{750}	Daily	0.1 Acre ponds, PBRs
AFDW	Daily	0.1 Acre ponds, PBRs
Pulse Amplitude Modulation (PAM; Photosynthetic efficiency)	Daily	0.1 Acre ponds, PBRs
Lipid Content (FAME)	Daily	0.1 Acre ponds, PBRs
Total Organic Carbon (TOC)	Daily	0.1 Acre ponds, PBRs
Microscopy	Bi-weekly	0.1 Acre ponds, PBRs
Microbial Ecology	Bi-weekly	0.1 Acre ponds
pH Measurement	10 minutes	0.1 Acre ponds, PBRs
Water Temperature	10 minutes	0.1 Acre ponds, PBRs
Dissolved Oxygen	10 minutes	0.1 Acre ponds, PBRs
Conductivity	10 minutes	0.1 Acre ponds, PBRs
Air Temperature	10 minutes	Weather Station
Wind Speed	10 minutes	Weather Station
Wind Direction	10 minutes	Weather Station
Photosynthetically Active Radiation	10 minutes	Weather Station
Precipitation	10 minutes	Weather Station
Relative Humidity	10 minutes	Weather Station
Trap Pond Samples	Bi-weekly	378 L traps (see **Figure J1**)

Table G2: Summary of work activity and time involving subject strain. The proper personal protective equipment (PPE) assigned for each task is defined by the risk assessment process, is documented, and is shared with all workers along with training. PPE by activity is listed in **Table I1**.

Worker Activity	Protective Equipment	Number of Workers Exposed	Maximum Exposure (hrs/day)	Maximum Duration (days/year)
Scale-up of cultures	Proper PPE	3-4	4	52
Inoculation of ponds	Proper PPE	3-4	4	52
Sampling of ponds	Proper PPE	3-4	1	365
Sample processing (lab)	Proper PPE	3-4	2	365
Experimental termination	Proper PPE	3-4	4	52

Table H1: CAAF inlet water-quality periodic testing summary. Colorado River water is delivered to the site by the Imperial Irrigation District through an extensive agricultural canal system running throughout the Imperial Valley.

Measurement	Min	Max	Mean (n=8)
Phosphorus	ND	0.25	0.15
Nitrogen	ND	13.5	3.60
Nitrogen, TKN	ND	13.4	5.43
Nitrate/Nitrite	ND	0.54	0.28
Alkalinity	146	163	155
Salinity	2.0	2.0	2.0
Solids (TDS)	680	1010	758
Al	ND	3.01	2.17
As	ND	0.01	0.01
Ca	27.1	90.2	75.9
Fe	0.82	4.42	2.07
K	ND	5.69	5.56
Mg	10.7	34.1	29.2
Na	41.9	136	109
Si	4.60	27.0	11.41

Table I1: Checklist for SGI's implementation of the NIH Guidelines (NIH) for CAAF ponds subject to this application, and for the CAAF greenhouse and photobioreactors. Adapted from NIH appendix K – Table 1. Criterion details are found in NIH appendix K-VI-B.

	CRITERION	GLSP		BL1-LS	
		SGI Open Pond	NIH	SGI GH & PBR	NIH
1.	Formulate and implement institutional codes of practice for safety of personnel and adequate control of hygiene and safety measures.	✔	K-II-A	✔	G-I
2.	Provide adequate written instructions and training of personnel to keep work place clean and tidy and to keep exposure to biological, chemical or physical agents at a level that does not adversely affect health and safety of employees.	✔	K-II-B	✔	G-I
3.	Provide changing and hand washing facilities as well as protective clothing, appropriate to the risk, to be worn during work.	✔	K-II-C	✔	G-II-A-1-h
4.	Prohibit eating, drinking, smoking, mouth pipetting, and applying cosmetics in the work place.	✔	K-II-C	✔	G-II-A-1-d G-II-A-1-e
5.	Internal accident reporting.	✔	K-II-G	✔	K-III-A
6.	Medical surveillance.	**(1)**	NR	**(1)**	NR
7.	Viable organisms should be handled in a system that physically separates the process from the external environment (closed system or other primary containment).	**(2)**	NR	✔	K-III-B
8.	Culture fluids not removed from a system until organisms are inactivated.	**(3)**	NR	**(3)**	K-III-C
9.	Inactivation of waste solutions and materials with respect to their biohazard potential.	✔	K-II-E	✔	K-III-C
10.	Control of aerosols by engineering or procedural controls to prevent or minimize release of organisms during sampling from a system, addition of materials to a system, transfer of cultivated cells, and removal of material, products, and effluent from a system.	✔	Minimize Procedure K-II-F	✔	Minimize Engineer K-III-B K-III-D
11.	Treatment of exhaust gases from a closed system to minimize or prevent release of viable organisms.		NR	✔	Minimize K-III-E
12.	Closed system that has contained viable organisms not to be opened until sterilized by a validated procedure.		NR	✔	K-III-F
13.	Closed system to be maintained at as a low pressure as possible to maintain integrity of containment features.	✔	NR	✔	NR
14.	Rotating seals and other penetrations into closed system designed to prevent or minimize leakage.		NR		NR
15.	Closed system shall incorporate monitoring or sensing devices to monitor the integrity of containment.		NR		NR
16.	Validated integrity testing of closed containment system.		NR		NR
17.	Closed system to be permanently identified for record keeping purposes.		NR		NR
18.	Universal biosafety sign to be posted on each closed system.		NR		NR
19.	Emergency plans required for handling large losses of cultures.	✔	K-II-G	✔	K-III-G
20.	Access to the work place.	✔	NR	✔	G-II-A-1-a
21.	Requirements for controlled access area.	✔	NR	✔	NR

(1) SGI maintains both a CA Injury and Illness Prevention Program and a CA Hazardous Materials Business Plan to address medical surveillance activities.

(2) SGI's 0.1-acre ponds use bird netting in an abundance of caution. However, for larger ponds, bird netting will not likely be feasible and alternative measures and/or deterrents will be evaluated.

(3) Excluding sampling and bulk transfers to another pond / PBR having appropriate containment.

GH = greenhouse; PBR = photobioreactor; NR = not required

Table I2: List of worker tasks performed at CAAF

Worker Activity	Protective Equipment/ Engineering Controls	# of Workers Exposed	Maximum Duration (hours/day)	Maximum Duration (days/year)
Scale up culture: Preparation of media Cultivation in photobioreactors	Safety glasses / disposable nitrile gloves	3 – 4	4	52
Preparation and pond inoculation		3 – 4	4	52
Sample collection		3 – 4	1	365
Pond monitoring		3 – 4	1	365
Addition of water / nutrients		3 – 4	1	365
Experimental termination		3 – 4	4	52
Cleaning ponds (mini or large)	Safety glasses / PVC gloves, rubber boots	3 – 4	0.5 – 2	52
Laboratory activities – Algae cultivation	Safety glasses / disposable nitrile gloves	3 – 4	0.5	365
Laboratory activities – sample processing		3 – 4	2	365
Laboratory activities – general activities		3 – 4	2	365
Laboratory activities – analytical testing	Safety glasses / disposable nitrile gloves. Preparation done in hood	3 – 4	2	365

Table I3: Qualitative exposure potential for tasks carried out at CAAF.

Characteristics of Potential Exposure		
Task	**Description**	**Qualitative Assessment of Potential for Exposure**
Scale-up culture – media prep	Fill photobioreactors with filtered water, add appropriate amount of nutrients to provide desired conditions.	-5
Scale-up culture – inoculation of photobioreactor	With the use of pumps, transfer contents of growth bottles to photobioreactor	-2
Preparation and pond inoculation	Fill ponds with filtered water, add appropriate amount of nutrients to provide desired conditions. With the use of hoses and pumps, transfer contents of photobioreactor to the pond to be inoculated	-1
Sample collection	Collect samples by aspiration of contents of pond and algae traps and transfer into plastic vials	3
Bioaerosol sample collection	Utilizing air sampling pump, collect samples in a filter. Extract filter with kit and transport samples to lab for processing	-1
Pond monitoring	Walking around the facility to assess normal operation	2
Addition of water and/or nutrients	Depending on physico-chemical characteristics of growth media, add more water to keep levels constant (this is done by pumps and hoses)	1
Experimental termination	Addition of chlorine for elimination of algae. Drain tank to evaporation pond	2
Pond cleaning	Scrubbing of walls and floor of pond to eliminate any biological cell. Rinsing with water	0
Laboratory activities – Algae cultivation	Growth room procedures: Inoculation of flasks and /or carboys with algal cultures Preparation and addition of nutrient mix Monitoring of growth room conditions	-2
Laboratory activities – Sample processing	In open room and with automatic equipment, transfer of algae from sample vials to smaller vials or plates for Sample preparation through centrifuging and concentration. Process air and pond samples for qPCR analysis and ??	-3
Laboratory activities – general activities	Miscellaneous activities associated with the analytical tests and growth room: Preparation of growth media / Sterilization of materials in autoclave / Calibration of instruments / Cleaning equipment / Discard samples	-6
Laboratory activities – analytical tests	In open room and with automatic pipettes, carry out analytical tests: Determination of optical density / Observation under microscope / Lyophilization of algae / Determination of salinity / Determination of ash-free weight	-1

Table I4: Qualitative risk assessment for the worker population.

Hazard Category (Highest → Lowest/None)	Qualitative Rating for Exposure Potential: Highest				Lowest/None
I					
II					
III					
IV			sample collection	pond monitoring / experiment termination	pond cleaning

Table I5: Qualitative risk assessment for the environment.

Hazard Category (Highest → Lowest/None)	Qualitative Rating for Exposure Potential (Highest → Lowest/None)				
I					
II					
III					
IV		aquatic plants, benthos, fish, zooplankton, reptiles, amphibians	riparian birds, riparian mammals, terrestrial insects	upland mammals, upland birds, terrestrial plants, soil organisms	

Table I6: *Parachlorella* inactivation study results.

Strain	4.0% Na hypochlorite application	Exposure Time		
		1 hour	2 hour	4 hours
STR00010	0 mL/L	+	+	+
	1 mL/L	+	+	+
	2 mL/L	-	-	-
	4 mL/L	-	-	-
STR00012	0 mL/L	+	+	+
	1 mL/L	-	-	-
	2 mL/L	-	-	-
	4 mL/L	-	-	-
STR26155	0 mL/L	+	+	+
	1 mL/L	-	-	-
	2 mL/L	-	-	-
	4 mL/L	-	-	-

"+" growth observed
"-" growth NOT observed therefore the culture was inactivated
Final concentrations of hypochlorite used were 0%, 0.004%, 0.008%, and 0.016%

Table I7: Qualitative risk assessment for the general population.

Hazard Category (Highest → Lowest/None)	Qualitative Rating for Exposure Potential: Highest				Lowest/None
I					
II					
III					
IV			extreme weather event[1]		fence-line exposure

[1]During a rare, but extreme weather event with no warning, dispersion of the microalgae may warrant a higher Exposure Factor Rating. Even in the worst-case scenario of moderate potential for exposure, the potential for a health risk (i.e. allergic reaction) is negligible due to the dispersion effect.

Table J1: Detail of sample type, methodology, and endpoint assay, for environmental monitoring.

Sample Type	Monitoring Methodology	Frequency	Duration	Primary Molecular Assay	Seconday Molecular Assay
Bio-aerosols	active	weekly	~ 3 months	qPCR	NA
Algae traps	active	bi-weekly	~ 3 months	microbiome profiling	qPCR for all samples where Parachlorella is detected
CAAF ponds	active	bi-weekly	~ 3 months	qPCR (with periodic microbiome profiling)	NA
Environmental stations (soil and water)	passive	monthly	1 year from start of first outdoor culture	microbiome profiling	qPCR for all samples where Parachlorella is detected